

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
5 February 2004 (05.02.2004)

PCT

(10) International Publication Number
WO 2004/011651 A1

(51) International Patent Classification⁷: C12N 15/50, C07K 14/165, G01N 33/50, C12Q 1/68, C12N 7/00

(74) Agent: MILES, John, S.; Eric Potter Clarkson, Park View House, 58 The Ropewalk, Nottingham NG1 5DD, United Kingdom (GB).

(21) International Application Number:

PCT/GB2003/002832

(22) International Filing Date: 1 July 2003 (01.07.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0217434.0 27 July 2002 (27.07.2002) GB

(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(71) Applicant (for all designated States except US): THE ROYAL VETERINARY COLLEGE [GB/GB]; University of London, Royal College Street, London NW1 0TU (GB).

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(72) Inventors; and

(75) Inventors/Applicants (for US only): BROWNLIE, John [GB/GB]; Royal veterinary college, Hawkshead Lane, North Mymms, Hartfield, Herts AL9 7TA (GB). CHALKER, Victoria, Jane [GB/GB]; Royal veterinary college, Hawkshead Lane, North Mymms, Hartfield, Herts AL9 7TA (GB). ERLES, Kerstin [DE/GB]; Royal veterinary college, Hawkshead Lane, North Mymms, Hartfield, Herts AL9 7TA (GB).

Published:

— with international search report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.



WO 2004/011651 A1

(54) Title: CANINE RESPIRATORY CORONAVIRUS (CRCV) SPIKE PROTEIN, POLYMERASE AND HEMAGGLUTININ/ESTERASE

ctcagatgaa tttgaaatat gctatttagtg ctaagaatag agcccgcact gttgctggtg	60
tttccatact tagtactatg actggcagaa tgtttcatca aaaatgtttg aaaagtatag	120
cagctacacg tggtgttcct gttgttatag gcaccactaa atttatggc ggctggatg	180
atatgttacg tcgccttatt aaagatgtt acaatcctgt acttatgggt tgggattatc	240
ctaagtgtga	250

(57) Abstract: A canine respiratory coronavirus (CRCV) that is present in the respiratory tract of dogs with canine infectious respiratory disease and which has a low level of homology to the enteric canine coronavirus, but which has a high level of homology to all bovine coronavirus strains (eg Quebec and LY138) and human coronavirus strain OC43. The CRCV spike, polymerase and hemagglutinin/esterase cDNA and protein partial sequences are listed in Figures (1) to (4), (13) and (14).

**CANINE RESPIRATORY CORONAVIRUS (CRCV)
SPIKE PROTEIN, POLYMERASE AND
HEMAGGLUTININ/ESTERASE**

The present invention relates to biological material, and in particular to a canine respiratory coronavirus that is present in dogs having canine infectious respiratory disease.

5 Canine infectious respiratory disease (CIRD) is a highly contagious disease common in dogs housed in crowded conditions such as re-homing centres and boarding or training kennels. Many dogs suffer only from a mild cough and recover after a short time, however in some cases a severe bronchopneumonia can develop (Appel and Binn, 1987).

10 The pathogenesis of CIRD is considered to be multifactorial, involving several viruses and bacteria. The infectious agents considered to be the major causative pathogens of CIRD are canine parainfluenzavirus (CPIV) (Binn *et al.*, 1967), canine adenovirus type 2 (CAV-2) (Ditchfield *et al.*, 1962) and the bacterium *Bordetella bronchiseptica* (Bemis *et al.*, 1977, Keil *et al.*, 1998). Also, canine herpesvirus, human reovirus and mycoplasma species have been isolated from dogs with symptoms of CIRD (Karpas *et al.*, 1968, Lou and Wenner 1963, Randolph *et al.*, 1993) Additional factors like stress may also be important.

20 CIRD is rarely fatal but it delays re-homing of dogs at rescue centres and it causes disruption of schedules in training kennels as well as considerable treatment costs.

25 Vaccines are available against some of the infectious agents associated with this disease, namely *Bordetella bronchiseptica* as well as CPIV and CAV-2. However, despite the use of these vaccines, CIRD is still prevalent in kennels world-wide, which is possibly due to the vaccines not providing protection against all the infectious agents involved in CIRD.

We have discovered a novel coronavirus, which we have called canine respiratory coronavirus (CRCV), in a large kennelled dog population with a history of endemic respiratory disease, and we have shown that this virus is associated with CIRD.

5 Some members of the family *coronaviridae* are known to cause respiratory disease in humans, cattle, swine and poultry (Mäkelä *et al.*, 1998, Pensaert *et al.*, 1986, Ignjatovic and Sapats 2000). For example, bovine respiratory coronavirus is associated with shipping fever in cattle which is a multifactorial respiratory disease (Storz *et al.*, 2000).

10 However, coronaviruses were not suspected to have a role in the pathogenesis of CIRD. Indeed, with only a single exception, canine coronaviruses have been reported to be enteric viruses and to cause acute diarrhoea mainly in young dogs (for example, Tennant *et al.*, 1993). In a large study of viruses involved in canine respiratory diseases, Binn *et al.*
15 (1979) reported the detection of a canine coronavirus in the lung of a single dog that was also infected with SV5 and canine adenovirus 2, two other viruses that are associated with canine respiratory disease.

There are 30-40 dog vaccines commercially available in the UK for use against a number of pathogens that can cause a range of diseases, such as
20 neurological, enteric, hepatic and respiratory diseases. Most of these vaccines contain microbial agents such as Distemper virus, Canine Adenovirus-2, Canine parvovirus, canine parainfluenza virus and *Leptospira canicola* and *L. icteroohaemorrhagiae*. None of these vaccines contain canine coronaviruses.

25 The dog vaccines for use against canine respiratory diseases are marketed as vaccines for "kennel-cough" (see below). All of the vaccines contain

Bordetella bronchisepticum, which is a bacterium associated with “kennel cough”.

Coyne M.J. & May S.W., (1995) in their article entitled “Considerations in using a canine coronavirus vaccine” (published as a Pfizer Technical 5 Bulletin on the Internet at <http://www.pfizer.com/ah/vet/tref/trbull/ccv.html>), lists over 20 commercially available vaccines against either canine coronaviruses alone or against canine coronaviruses together with other organisms. Each of these vaccines is for canine enteric disease, and there is no suggestion that a canine coronavirus may be associated with 10 respiratory disease.

US Patents Nos. 6,057,436 and 6,372,224, both to Miller *et al* and assigned to Pfizer, Inc., describe the spike gene of the enteric canine coronavirus and uses therefor, including its use as a vaccine against gastroenteritis. Neither of these two patents suggest that a canine coronavirus may be involved in 15 CIRD.

Members of the family *coronaviridae* are enveloped viruses, 80-160nm in diameter, containing a linear positive-stranded RNA genome. The structural proteins of coronaviruses are the spike glycoprotein (S), the membrane glycoprotein (M) and the nucleocapsid protein (N). The hemagglutinin/esterase glycoprotein (HE) is found only on the surface of 20 group II coronaviruses (e.g. bovine coronavirus and murine hepatitis virus) (Spaan *et al*, 1988). Further details of the structure of coronaviruses may be found in the chapter by Cavanagh *et al* entitled “Coronaviridae” p407-411, in “Virus Taxonomy, 6th Report of the International Committee on Taxonomy 25 of Viruses”, pub. Springer-Verlag Wein, New York, Eds. Murphy *et al*, which is incorporated herein by reference.

The canine respiratory coronavirus (CRCV) of the invention may be characterised as a coronavirus present in the respiratory tracts of dogs with infectious respiratory disease. To further characterise CRCV, we have determined the sequence of 250 nucleotide residues of the CRCV polymerase (pol) cDNA (Figure 1 and SEQ ID NO: 1) which corresponds to an 83 amino acid partial sequence of the pol protein (Figure 2 and SEQ ID NO: 2). We have also cloned and determined the sequence of the 4092 nucleotide residues of the CRCV spike (S) cDNA (Figure 3 and SEQ ID NO: 3), corresponding to 1363 amino acids (Figure 4 and SEQ ID NO: 4).
5 We have also determined the sequence of 497 nucleotide residues of the CRCV hemagglutinin/esterase (HE) gene (Figure 13 and SEQ ID NO: 21), corresponding to 165 amino acids (Figure 14 and SEQ ID NO: 22). We have identified that CRCV has a surprisingly low homology to the enteric canine coronavirus (CCV) while it has an unexpectedly high level of
10 homology to bovine coronavirus (strain LY138 or Quebec) and human coronavirus (strain OC43).
15

A culture of "Spike D-1 CRCV", which is XL1-Blue *E. coli* (Stratagene) containing a pT7Blue2 plasmid (Novagen) whose insert contains a portion of the CRCV spike cDNA, has been deposited under the Budapest Treaty at
20 NCIMB Ltd under Accession number NCIMB 41146 on 25 July 2002. The depositor of NCIMB 41146 is the Royal Veterinary College, Royal College Street, London NW1 OTU, UK. The address of NCIMB Ltd is 23 St. Machar Drive, Aberdeen, Scotland, AB24 3RY, UK.

The phylogenetic relationship of CRCV to eleven known coronaviruses was
25 determined based upon a comparison of the 250 nucleotide sequence from the CRCV pol gene and the corresponding regions of the other viruses (Figure 5). The bovine coronavirus (BCV), human coronavirus (HCV) strain OC43 and hemagglutinating encephalomyelitis virus (HEV) were

found to be most closely related to CRCV, while the enteric CCV was found to be only distantly related to CRCV.

Over the 250 sequenced residues of the pol cDNA, corresponding to 83 amino acids, CRCV has only 68.5% and 75.9% sequence identity at the 5 nucleotide and amino acid levels, respectively, with the equivalent region of the enteric CCV (strain 1-71) pol gene (Genbank Accession No. AF124986), as shown in Figure 6 and 7.

Over the 4092 sequenced nucleotide residues of the CRCV S gene, corresponding to 1363 amino acids, CRCV has 45% and 21.2% sequence 10 identity at the nucleotide (Figure 8) and amino acid levels, respectively, with the equivalent region of the enteric CCV (strain 1-71) S gene.

Enteric CCV is not a group II coronavirus and does not possess an HE gene, hence it is not possible to determine the extent of sequence identity between this gene in CRCV and in enteric CCV.

15 Except as described below, the percentage identity between two nucleotide or two amino acid sequences was determined using FASTA version 34 (Pearson WR. (1990) "Rapid and sensitive sequence comparison with FASTP and FASTA". *Methods Enzymol.*;183:63-98). FASTA settings were Gap open penalty -16 and Gap extension penalty -4.

20 The percentage identity between the CRCV and enteric CCV spike sequences was determined using GCG version 10 (Genetics Computer Group, (1991), Program Manual for the GCG Package, Version 7, April 1991, 575 Science Drive, Madison, Wisconsin, USA 53711). The GCG parameters used were: Gap creation penalty 50, gap extension penalty 3 for 25 DNA, and Gap creation penalty 8 and Gap extension penalty 2 for Protein.

Sequence alignments were performed using ClustalX (Thompson *et al.*, 1997).

By contrast, over the 250 sequenced residues of the pol cDNA, CRCV has 98.8% sequence identity with the equivalent region of the BCV strain 5 Quebec pol gene (Genbank Accession No. AF220295), 98.4% sequence identity with the BCV strain LY138 pol gene (Genbank Accession No. AF124985) and 98.4% sequence identity with the HCV OC43 pol gene (Genbank Accession No. AF124989).

There was only a single amino acid difference between the CRCV pol 10 protein over the 83 sequenced amino acids and the BCV, HCV and HEV pol proteins which is that CRCV has E (Glu) as opposed to D (Asp) at the position corresponding to position 4975 in the BCV genome (Accession No. SWALL: Q91A29). Thus the CRCV pol protein is 99% identical to the BCV, HCV and HEV pol proteins over this region.

15 The one and three letter amino acid codes of the IUPAC-IUB Biochemical Nomenclature Commission are used herein.

Over the 497 sequenced nucleotide residues, corresponding to 165 amino acids, of the HE gene, CRCV has 98.994% and 98.2% sequence identity 20 with the equivalent region of the BCV strain LY138 HE gene (Genbank Accession No. AF058942) at the nucleotide and amino acid levels respectively. CRCV has 98.189% (nucleotide) and 98.2% (amino acid) sequence identity with human enteric coronavirus (HECV) HE gene (Genbank Accession No. L07747); 97.4% (nucleotide) and 95.2% (amino acid) sequence identity with the HCV OC43 HE gene (Genbank Accession 25 No. M76373); and 92.0% (nucleotide) and 93.9% (amino acid) identity with HEV (Genbank Accession Nos. AF481863), as shown in Figures 15 and 16.

As shown in Figure 16 and Table 3, the three amino acids that are different between the CRCV HE protein and each of the BCV, HECV, HCV and HEV S proteins, within the 165 amino acids of the CRCV HE protein, are F (Phe) as opposed to L (Leu), N (Asn) as opposed to T (Thr), and L (Leu) as opposed to V (Val) at positions corresponding to position 235, 242 and 253, respectively, in the BCV, HECV, HCV OC43 and HEV HE genes (Figure 16). Thus F at position 235, N at position 242 and L at position 253 could be said to be CRCV HE protein-specific amino acids.

Over the 4092 sequenced nucleotide residues, corresponding to 1363 amino acids, of the CRCV S gene, CRCV has 97.3% and 96% identity with the equivalent region of BCV strain LY138 (Genbank Accession No. AF058942) at the nucleotide and amino acid levels respectively. CRCV has 96.9% (nucleotide) and 95.2% (amino acid) identity with HCV strain OC43 (Genbank Accession No. Z32768), and 83.8% (nucleotide) and 80.4% (amino acid) identity with HEV (Genbank Accession Nos. AF481863 (cDNA) and AAM 77000 (protein)) as shown in Figures 9 and 10.

The amino acids that are different between the CRCV S protein and each of the BCV, HCV and HEV S proteins, within the 1363 amino acids of the CRCV S protein, are listed in Table 1 below. Thus the amino acids listed in Table 1 could be said to be CRCV S protein-specific amino acids. The amino acids are numbered from the initial M residue at the start of the CRCV protein, as shown in Figure 4.

Table 1: List of 39 amino acids specific to the CRCV S protein that are not present in the BCV, HCV and HEV S proteins.

<u>Position</u>	<u>Amino acid</u>
103	V
118	V
166	D
171	M
179	K
192	P
210	S
235	H
267	F
388	F
407	M
436	S
440	I
447	I
501	F
525	Y
528	N
540	L
582	K
608	G

<u>Position</u>	<u>Amino acid</u>
692	G
695	S
757	W
758	G
763	Q
769	T
786	P
792	H
818	R
827	P
828	V
887	F
933	D
977	F
1011	T
1018	S
1063	K
1256	L
1257	M

A first aspect of the invention provides a coronavirus S protein, or fragment thereof, having at least 75% amino acid sequence identity with the CRCV S protein whose amino acid sequence is listed in Figure 4, and having at least one of V at position 103; V at position 118; D at position 166; M at position 5 171; K at position 179; P at position 192; S at position 210; H at position 235; F at position 267; F at position 388; M at position 407; S at position 436; I at position 440; I at position 447; F at position 501; Y at position 525; N at position 528; L at position 540; K at position 582; G at position 608; G at position 692; S at position 695; W at position 757; G at position 758; Q at 10 position 763; T at position 769; P at position 786; H at position 792; R at position 818; P at position 827; V at position 828; F at position 887; D at position 933; F at position 977; T at position 1011; S at position 1018; K at position 1063; L at position 1256; and M at position 1257. The amino acids are numbered from the initial M at the start of the CRCV S protein, as listed 15 in Figure 4 (SEQ ID NO: 4).

It is appreciated that the partial nucleotide sequence of CRCV S can be readily determined by a person of ordinary skill in the art by sequencing the insert of the plasmid contained in *E. coli* strain D-1 CRCV, that has been deposited under the Budapest Treaty at NCIMB Ltd. under Accession 20 number NCIMB 41146 on 25 July 2002. Furthermore, this DNA can be used as a hybridisation probe, or as the basis for the design of probes, in the isolation of CRCV nucleic acid in dogs.

For the avoidance of doubt, the invention includes a coronavirus S protein, or fragment thereof, having at least 75% amino acid sequence identity with the CRCV S protein (SEQ ID NO: 4), and comprising at least one of the amino acids specific for the CRCV S protein at the position listed in Table 25 1.

By "protein" we also include the meaning glycoprotein. The amino acid sequence of a glycoprotein refers to the amino acid sequence of the polypeptide backbone of the glycoprotein, irrespective of the type, number, sequence and position of the sugars attached thereto.

5 Typically, the invention includes an isolated or recombinant protein, and not an unmodified CRCV protein present as a CRCV component.

The invention includes a coronavirus S protein, or fragment thereof, having at least 76% amino acid sequence identity with the CRCV S protein (SEQ ID NO: 4), or at least 77%, or at least 78%, or at least 79%, or at least 80%,
10 or at least 81%, or at least 82%, or at least 83%, or at least 84%, or at least 85%, or at least 86%, or at least 87%, or at least 88%, or at least 89%, or at least 90%, or at least 91%, or at least 92%, or at least 93%, or at least 94%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 99% amino acid sequence identity with the CRCV S protein, and
15 comprising at least one of the amino acids specific for the CRCV S protein at the position listed in Table 1.

The invention also includes a coronavirus S protein, or fragment thereof, having at least 75%, or at least 80%, or at least 85% or at least 90% or at least 95% amino acid sequence identity with the CRCV S protein (SEQ ID NO: 4), and comprising at least 2, or at least 3, or at least 4, or at least 5, or at least 6, or at least 7, or at least 8, or at least 9, or at least 10, or at least 11, or at least 12, or at least 13, or at least 14, or at least 15, or at least 16, or at least 17, or at least 18, or at least 19, or at least 20, or at least 21, or at least 22, or at least 23, or at least 24, or at least 25, or at least 26, or at least 27, or at least 28, or at least 29, or at least 30, or at least 31, or at least 32, or at least 33, or at least 34, or at least 35, or at least 36, or at least 37, or at least 38 of the amino acids specific for CRCV S protein at the positions listed in Table 1.

Preferably, the coronavirus S protein, or fragment thereof comprises all 39 of the amino acid residues specific for CRCV S protein at the positions listed in Table 1.

Thus the invention includes a BCV, HCV or HEV S protein or fragment thereof, that has been modified at at least one position listed in Table 1 to resemble the CRCV S protein.

Preferably, the coronavirus S protein of the invention is a CRCV S protein that comprises or consists of the sequence listed in Figure 4 (SEQ ID NO: 4), or a variant thereof with at least 97% identity with the sequence listed in Figure 4. Preferably, the variant has at least 98%, or at least 99% amino acid sequence identity with the sequence listed in Figure 4. More preferably the variant has at least 99.1%, or at least 99.2%, or at least 99.3%, or at least 99.4%, or at least 99.5%, or at least 99.6%, or at least 99.7%, or at least 99.8%, or at least 99.9% amino acid sequence identity with the sequence listed in Figure 4.

Thus the variant of the coronavirus S protein of the invention includes a protein that comprises or consists of the sequence listed in Figure 4 (SEQ ID NO: 4) but has between 1 and 40 amino acid differences from the sequence listed in Figure 4. Preferably, the variant has less than 40 amino acid differences from the sequence listed in Figure 4. More preferably the variant has less than 35, less than 30, or less than 25, or less than 20, or less than 15, or 10 or 9 or 8 or 7 or 6 or 5 or 4 or 3 or 2 amino acid differences, or a single amino acid difference, from the sequence listed in Figure 4.

The invention also includes a CRCV S protein fragment comprising a fragment of the sequence listed in Figure 4 (SEQ ID NO: 4) which comprises at least one of the amino acids specific for CRCV S protein at the position listed in Table 1.

The invention includes a coronavirus S protein, or fragment thereof, having at least 75% amino acid sequence identity with BCV strain LY138 S protein (SEQ ID NO: 14, Genbank Accession No. AF058942), and comprising at least one of V at position 103; V at position 118; D at position 166; M at 5 position 171; K at position 179; P at position 192; S at position 210; H at position 235; F at position 267; F at position 388; M at position 407; S at position 436; I at position 440; I at position 447; ; F at position 501; Y at position 525; N at position 528; L at position 540; K at position 582; G at position 608; G at position 692; S at position 695; W at position 757; G at 10 position 758; Q at position 763; T at position 769; P at position 786; H at position 792; R at position 818; P at position 827; V at position 828; F at position 887; D at position 933; F at position 977; T at position 1011; S at position 1018; K at position 1063; L at position 1256 and M at position 1257.

15 For the avoidance of doubt, the invention includes a coronavirus S protein, or fragment thereof, having at least 75% amino acid sequence identity with BCV strain LY138 S protein (SEQ ID NO: 14), and comprising at least one of the amino acids specific for CRCV S protein at the position listed in Table 1.

20 The invention includes a coronavirus S protein, or fragment thereof, having at least 76% amino acid sequence identity with BCV strain LY138 S protein, or at least 77%, or at least 78%, or at least 79%, or at least 80%, or at least 81%, or at least 82%, or at least 83%, or at least 84%, or at least 85%, or at least 86%, or at least 87%, or at least 88%, or at least 89%, or at 25 least 90%, or at least 91%, or at least 92%, or at least 93%, or at least 94%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 99% amino acid sequence identity with BCV strain LY138 S protein, and

having at least one of the amino acids specific for CRCV S protein at the position listed in Table 1.

The invention also includes a coronavirus S protein, or fragment thereof, having at least 75%, or at least 80%, or at least 85%, or at least 90%, or at 5 least 95% amino acid sequence identity with BCV strain LY138 S protein (SEQ ID NO: 14), and comprising at least 2, or at least 3, or at least 4, or at least 5, or at least 6, or at least 7, or at least 8, or at least 9, or at least 10, or at least 11, or at least 12, or at least 13, or at least 14, or at least 15, or at 10 least 16, or at least 17, or at least 18, or at least 19, or at least 20, or at least 21, or at least 22, or at least 23, or at least 24, or at least 25, or at least 26, or at least 27, or at least 28, or at least 29, or at least 30, or at least 31, or at least 32, or at least 33, or at least 34, or at least 35, or at least 36, or at least 37, or at least 38 of the amino acids specific for CRCV S protein at the positions listed in Table 1.

15 Preferably, the coronavirus S protein, or fragment thereof comprises all 39 of the amino acid residues specific for CRCV S protein at the positions listed in Table 1.

A second aspect of the invention provides a coronavirus pol protein, or fragment thereof, having at least 90% amino acid sequence identity with the 20 BCV pol protein (SEQ ID NO: 5) and comprising the amino acid E at the position corresponding to position 4975 in the BCV genome (Accession No. SWALL: Q91A29).

The invention includes a coronavirus pol protein, or fragment thereof, having at least 91% amino acid sequence identity with BCV strain LY138 25 pol protein, or at least 92%, or at least 93%, or at least 94%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 99% amino acid sequence identity with BCV strain LY138 pol protein, and having the amino

acid E at the position corresponding to position 4975 in the BCV genome (Accession No. SWALL: Q91A29).

Preferably, the coronavirus pol protein, or fragment thereof is a CRCV pol protein or fragment thereof that comprises or consists of the amino acid sequence listed in Figure 2.

Thus the invention includes a BCV, HCV or HEV pol protein or fragment thereof, that has been modified at the amino acid corresponding to position 4975 in the BCV genome, to resemble the CRCV pol protein.

The invention also includes a CRCV pol protein fragment comprising a fragment of the sequence listed in Figure 2 (SEQ ID NO: 2) and having the amino acid E at the position corresponding to position 4975 in the BCV genome.

A third aspect of the invention provides a coronavirus HE protein, or fragment thereof, having at least 90% amino acid sequence identity with the BCV LY138 HE protein (Genbank Accession No. AF058942), and having at least one of F at position 235; N at position 242; and L at position 253. The amino acid positions are numbered from the initial M (which is number 1) at the start of the BCV HE protein.

The invention includes a coronavirus HE protein, or fragment thereof, having at least 91% amino acid sequence identity with BCV strain LY138 HE protein, or at least 92%, or at least 93%, or at least 94%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 99% amino acid sequence identity with BCV strain LY138 HE protein, and having at least one of F at position 235; N at position 242; and L at position 253. The amino acid positions are numbered from the initial M (which is number 1) at the start of the BCV HE protein.

The invention also includes a coronavirus HE protein, or fragment thereof, having at least 90%, or at least 91%, or at least 92%, or at least 93%, or at least 94%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 99% amino acid sequence identity with BCV strain LY138 HE protein, and having two of F at position 235; N at position 242; and L at position 253. The amino acid positions are numbered from the initial M (which is number 1) at the start of the BCV HE protein.

The invention further includes a coronavirus HE protein, or fragment thereof, having at least 90%, or at least 91%, or at least 92%, or at least 93%, or at least 94%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 99% amino acid sequence identity with BCV strain LY138 HE protein, and having all three of F at position 235; N at position 242; and L at position 253. The amino acid positions are numbered from the initial M (which is number 1) at the start of the BCV HE protein.

15 Preferably, the coronavirus HE protein, or fragment thereof is a CRCV HE protein or fragment thereof that comprises or consists of the amino acid sequence listed in Figure 14 (SEQ ID NO: 22).

Thus the invention includes a BCV, HCV, HECV or HEV HE protein or fragment thereof, that has been modified at one or more of the amino acids corresponding to position 235, 242; and 253 to resemble the CRCV HE protein.

The invention also includes a CRCV HE protein fragment comprising a fragment of the sequence listed in Figure 14 (SEQ ID NO: 22) and having one or more of the amino acid F at position 235, N at position 242, and L at position 253. The numbering of these amino acid positions corresponds to that of BCV LY138 HE protein (Genbank Accession No. AF058942) in

which residue number 1 is the initial M at the start of the BCV LY138 HE protein.

The coronavirus S, pol and HE proteins as defined above in the first, second and third aspects of the invention may be termed herein “CRCV” or
5 “CRCV-like” proteins.

A “CRCV S protein” is an S protein or fragment thereof that has the native CRCV S amino acid sequence as listed in Figure 4 (SEQ ID NO: 4), or a fragment thereof which comprises at least one of the amino acids specific for a CRCV S protein at the positions listed in Table 1.

10 A “CRCV pol protein” is a pol protein or fragment thereof that has the native CRCV pol amino acid sequence as listed in Figure 2 (SEQ ID NO: 2), or a fragment thereof which comprises the amino acid E at the position corresponding to position 4975 in the BCV genome.

15 A “CRCV HE protein” is an HE protein or fragment thereof that has the native CRCV HE amino acid sequence as listed in Figure 14 (SEQ ID NO: 22), or a fragment thereof which comprises one or more of the amino acid F at position 235, N at position 242, and L at position 253. The numbering of these amino acid positions corresponds to that of BCV LY138 HE protein
20 (Genbank Accession No. AF058942) in which residue number 1 is the initial M at the start of the BCV LY138 HE protein.

25 A “CRCV-like S protein” is an S protein or fragment thereof that does not have an amino acid sequence identical to the native CRCV S amino acid sequence (Figure 4 and SEQ ID NO: 4), but has at least 75% sequence identity with the corresponding region of the CRCV or BCV strain LY138 S protein, and has at least one of the amino acids specific for a CRCV S protein at the positions listed in Table 1.

A “CRCV-like S protein” also includes an S protein that does not have an amino acid sequence identical to the native CRCV S amino acid sequence (Figure 4 and SEQ ID NO: 4), but that comprises or consists of a variant of the sequence listed in Figure 4 with at least 97% identity with the sequence listed in Figure 4. Preferably, the variant has at least 98%, or at least 99% amino acid sequence identity with the sequence listed in Figure 4. More preferably the variant has at least 99.1%, or at least 99.2%, or at least 99.3%, or at least 99.4%, or at least 99.5%, or at least 99.6%, or at least 99.7%, or at least 99.8%, or at least 99.9% amino acid sequence identity with the sequence listed in Figure 4.

A “CRCV-like pol protein” is a pol protein or fragment thereof that does not have an amino acid sequence identical to the native CRCV pol amino acid sequence, but has at least 90% sequence identity with the corresponding BCV strain LY138 pol protein, and which has an E at the position corresponding to position 4975 in the BCV genome.

A “CRCV-like HE protein” is an HE protein or fragment thereof that does not have an amino acid sequence identical to the native CRCV HE amino acid sequence, but has at least 90% sequence identity with the corresponding BCV strain LY138 HE protein, and which has one or more of the amino acid F at position 235, N at position 242, and L at position 253. The numbering of these three amino acid positions corresponds to that of BCV LY138 HE protein (Genbank Accession No. AF058942) in which residue number 1 is the initial M at the start of the BCV LY138 HE protein.

Preferably, the CRCV or CRCV-like protein, or fragment thereof, is at least 25 10 amino acids in length. More preferably, the CRCV or CRCV-like protein, or fragment thereof, is at least 20, or at least 30, or at least 40, or at least 50, or at least 100, or at least 200, or at least 300, or at least 400, or at

least 500, or at least 600, or at least 700, or at least 800, or at least 900, or at least 1,000, or at least 1,100, or at least 1,200 amino acids in length.

Preferably, the CRCV or CRCV-like protein, or fragment thereof, is less than about 1,300 amino acids in length. More preferably, the CRCV or CRCV-like protein, or fragment thereof, is less than about 1,200, or less than about 1,100, or less than about 1,000, or less than about 900, or less than about 800, or less than about 700, or less than about 600, or less than about 500, or less than about 400, or less than about 300, or less than about 200, or less than about 100, or less than about 50 amino acids in length.

CRCV proteins may be isolated from CRCV, or may be made using protein chemistry techniques for example using partial proteolysis of isolated proteins (either exolytically or endolytically), or by *de novo* synthesis. Alternatively, the CRCV proteins, as well as CRCV-like proteins, may be made by recombinant DNA technology. Suitable techniques for cloning, manipulation, modification and expression of nucleic acids, and purification of expressed proteins, are well known in the art and are described for example in Sambrook *et al* (2001) "*Molecular Cloning, a Laboratory Manual*", 3rd edition, Sambrook *et al* (eds), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA, incorporated herein by reference.

Shorter fragments of CRCV and CRCV-like proteins, *ie* peptides, may be synthesised using standard techniques. Peptides may be synthesised by the Fmoc-polyamide mode of solid-phase peptide synthesis as disclosed by Lu *et al* (1981) *J. Org. Chem.* **46**, 3433 and references therein. Temporary N-amino group protection is afforded by the 9-fluorenylmethyloxycarbonyl (Fmoc) group. Repetitive cleavage of this highly base-labile protecting group is effected using 20% piperidine in N,N-dimethylformamide. Side-chain functionalities may be protected as their butyl ethers (in the case of serine

threonine and tyrosine), butyl esters (in the case of glutamic acid and aspartic acid), butyloxycarbonyl derivative (in the case of lysine and histidine), trityl derivative (in the case of cysteine) and 4-methoxy-2,3,6-trimethylbenzenesulphonyl derivative (in the case of arginine). Where glutamine or asparagine are C-terminal residues, use is made of the 4,4'-dimethoxybenzhydryl group for protection of the side chain amido functionalities. The solid-phase support is based on a polydimethylacrylamide polymer constituted from the three monomers dimethylacrylamide (backbone-monomer), bisacryloylethylene diamine (cross linker) and acryloylsarcosine methyl ester (functionalising agent). The peptide-to-resin cleavable linked agent used is the acid-labile 4-hydroxymethyl-phenoxyacetic acid derivative. All amino acid derivatives are added as their preformed symmetrical anhydride derivatives with the exception of asparagine and glutamine, which are added using a reversed N,N-dicyclohexyl-carbodiimide/1-hydroxybenzotriazole mediated coupling procedure. All coupling and deprotection reactions are monitored using ninhydrin, trinitrobenzene sulphonic acid or isotin test procedures. Upon completion of synthesis, peptides are cleaved from the resin support with concomitant removal of side-chain protecting groups by treatment with 95% trifluoroacetic acid containing a 50% scavenger mix. Scavengers commonly used are ethanedithiol, phenol, anisole and water, the exact choice depending on the constituent amino acids of the peptide being synthesised. Trifluoroacetic acid is removed by evaporation *in vacuo*, with subsequent trituration with diethyl ether affording the crude peptide. Any scavengers present are removed by a simple extraction procedure which on lyophilisation of the aqueous phase affords the crude peptide free of scavengers. Reagents for peptide synthesis are generally available from Calbiochem-Novabiochem (UK) Ltd, Nottingham NG7 2QJ, UK. Purification may be effected by any one, or a combination of, techniques such as size exclusion chromatography, ion-exchange chromatography and (principally) reverse-phase high performance

liquid chromatography. Analysis of peptides may be carried out using thin layer chromatography, reverse-phase high performance liquid chromatography, amino-acid analysis after acid hydrolysis and by fast atom bombardment (FAB) mass spectrometric analysis.

5 A fourth aspect of the invention provides a polynucleotide that encodes a CRCV or CRCV-like S, pol or HE protein according to the first, second and third aspects of the invention, or the complement thereof.

Preferably, the polynucleotide encodes a CRCV S protein according to the first aspect of the invention, or the complement thereof.

10 More preferably, the polynucleotide encoding the CRCV S protein comprises or consists of the sequence listed in Figure 3 (SEQ ID NO: 3).

It is appreciated that the sequence listed in Figure 3 (SEQ ID NO: 3) contains a Y at position 3531, which refers to either C or T. In both cases the corresponding amino acid is Ile. Thus the invention includes a 15 polynucleotide encoding a CRCV S protein which comprises or consists of the sequence listed in Figure 3, and having C at position 3531. The invention also includes a polynucleotide encoding a CRCV S protein which comprises or consists of the sequence listed in Figure 3, and having T at position 3531.

20 The invention also includes a CRCV S polynucleotide comprising a fragment of the sequence listed in Figure 3 (SEQ ID NO: 3), that encodes a protein having at least one of the amino acids specific for CRCV S protein at the position listed in Table 1, or the complement thereof.

25 Preferably, the polynucleotide encoding the pol protein comprises or consists of the sequence listed in Figure 1 (SEQ ID NO: 1), or the complement thereof.

The invention also includes a CRCV pol polynucleotide comprising a fragment of the sequence listed in Figure 1 (SEQ ID NO: 1) that encodes a protein having E at the position corresponding to position 4975 in the BCV genome, or the complement thereof.

5 Preferably, the polynucleotide encoding the HE protein comprises or consists of the sequence listed in Figure 13 (SEQ ID NO: 21), or the complement thereof.

The invention also includes a CRCV HE polynucleotide comprising a fragment of the sequence listed in Figure 13 (SEQ ID NO: 21) that encodes 10 a protein having one or more of the amino acid F at position 235, N at position 242, and L at position 253. The numbering of these three amino acid positions corresponds to that of BCV LY138 HE protein (Genbank Accession No. AF058942) in which residue number 1 is the initial M at the start of the BCV LY138 HE protein.

15 The polynucleotides as defined above are referred to herein as CRCV or CRCV-like polynucleotides of the invention.

A “CRCV-like polynucleotide” is a polynucleotide that does not have a base sequence identical to all or a fragment of the native CRCV cDNA sequence as listed in Figures 1, 3 and 13 (SEQ ID NOS: 1, 3 and 21), but 20 that encodes a CRCV or CRCV-like S pol or HE protein as defined above, or the complement thereof.

The CRCV is a positive strand RNA virus. The polynucleotide of the invention may be DNA or RNA. The RNA may be positive or negative strand RNA. The DNA may be single or double stranded DNA.

25 Suitable techniques for cloning and sequencing a cDNA from a positive strand RNA virus such as CRCV are well known in the art and are

described for example in Sambrook *et al* 2001, incorporated herein by reference.

The CRCV or CRCV-like polynucleotides of the invention may be any suitable size. However, for certain purposes, such as probing or amplifying,
5 it is preferred if the nucleic acid has fewer than 3,000, more preferably fewer than 1000, more preferably still from 10 to 100, and in further preference from 15 to 30 base pairs (if the nucleic acid is double-stranded) or bases (if the nucleic acid is single stranded). As is described more fully below, single-stranded DNA oligonucleotides, suitable for use as
10 hybridisation probes or as primers in a polymerase chain reaction, are particularly preferred.

Oligonucleotides that can specifically amplify, or hybridise to CRCV S, pol or HE polynucleotides, as opposed to BCV, HCV, HEV or enteric CCV S, pol or HE polynucleotides, are particularly preferred. Suitable
15 oligonucleotides can be determined by a person of skill in the art by reference to the nucleotide sequence comparisons in Figures 6, 8, 9 and 15.

It is appreciated that the CRCV or CRCV-like oligonucleotides may, even under highly stringent conditions, hybridise to nucleic acid, whether RNA or DNA, from HCV, BCV, and HEV as well as from CRCV. However, it is preferred if the CRCV or CRCV-like oligonucleotides hybridise to nucleic acid from CRCV under more stringent conditions than to nucleic acid from HCV, BCV or HEV. This can either be determined experimentally or by a comparison of the oligonucleotide sequence with the respective CRCV, HCV, BCV and HEV sequences, as is well known to one of skill in the art
20 (Sambrook *et al* 2001).

It is also appreciated that the CRCV or CRCV-like oligonucleotides may hybridise to nucleic acid, whether RNA or DNA, from the enteric CCV as

well as from CRCV. However, it is preferred if the CRCV or CRCV-like oligonucleotides hybridise to nucleic acid from CRCV under more stringent conditions than to nucleic acid from enteric CCV. This can either be determined experimentally or by a comparison of the oligonucleotide sequence with the respective sequences, as is well known to one of skill in the art (Sambrook *et al* 2001). Preferably, the oligonucleotides do not hybridise to nucleic acid from enteric CCV at all under stringent conditions (see below).

Conveniently, the CRCV or CRCV-like polynucleotides or oligonucleotides further comprise a detectable label.

By "detectable label" is included any convenient radioactive label such as ^{32}P , ^{33}P or ^{35}S which can readily be incorporated into a nucleic acid molecule using well known methods; any convenient fluorescent or chemiluminescent label which can readily be incorporated into a nucleic acid is also included. In addition the term "detectable label" also includes a moiety which can be detected by virtue of binding to another moiety (such as biotin which can be detected by binding to streptavidin); and a moiety, such as an enzyme, which can be detected by virtue of its ability to convert a colourless compound into a coloured compound, or *vice versa* (for example, alkaline phosphatase can convert colourless *o*-nitrophenylphosphate into coloured *o*-nitrophenol). Conveniently, the nucleic acid probe may occupy a certain position in a fixed array and whether a nucleic acid hybridises to it can be determined by reference to the position of hybridisation in the fixed array.

Labelling with [^{32}P]dCTP may be carried out using a Rediprime[®] random primer labelling kit supplied by Amersham.

Primers which are suitable for use in a polymerase chain reaction (PCR; Saiki *et al* (1988) *Science* 239, 487-491) are preferred. Suitable PCR primers may have the following properties:

It is well known that the sequence at the 5' end of the oligonucleotide need
5 not match the target sequence to be amplified.

It is usual that the PCR primers do not contain any complementary structures with each other longer than 2 bases, especially at their 3' ends, as this feature may promote the formation of an artefactual product called "primer dimer". When the 3' ends of the two primers hybridise, they form a
10 "primed template" complex, and primer extension results in a short duplex product called "primer dimer".

Internal secondary structure should be avoided in primers. For symmetric PCR, a 40-60% G+C content is often recommended for both primers, with no long stretches of any one base. The classical melting temperature
15 calculations used in conjunction with DNA probe hybridization studies often predict that a given primer should anneal at a specific temperature or that the 72°C extension temperature will dissociate the primer/template hybrid prematurely. In practice, the hybrids are more effective in the PCR process than generally predicted by simple T_m calculations.

20 Optimum annealing temperatures may be determined empirically and may be higher than predicted. *Taq* DNA polymerase does have activity in the 37-55°C region, so primer extension will occur during the annealing step and the hybrid will be stabilised. The concentrations of the primers are equal in conventional (symmetric) PCR and, typically, within 0.1- to 1 μ M
25 range.

It will further be appreciated that if a control amplification reaction is to be carried out, for example using primers complementary to an ubiquitously expressed gene, that it may be beneficial for the products of the control and CRCV or CRCV-like products to be of different sizes, such that the two
5 products may be distinguished by the detection means employed, for example by mobility on agarose gel electrophoresis. However, it may be desirable for the two products to be of similar size, for example both between 100 and 1000, or between 100 and 600 nucleotides long. This may aid simultaneous analysis of the products, for example by gel
10 electrophoresis, and may also mean that the control and CRCV or CRCV-like amplification reactions may have similar performance characteristics, in terms, for example, of relative rates of accumulation of product at different stages during the reaction.

Any of the nucleic acid amplification protocols can be used in the method
15 of the invention including the polymerase chain reaction, QB replicase and ligase chain reaction. Also, NASBA (nucleic acid sequence based amplification), also called 3SR, can be used as described in Compton (1991) *Nature* 350, 91-92 and AIDS (1993), Vol 7 (Suppl 2), S108 or SDA (strand displacement amplification) can be used as described in Walker *et al*
20 (1992) *Nucl. Acids Res.* 20, 1691-1696. The polymerase chain reaction is particularly preferred because of its simplicity.

When a pair of suitable nucleic acids of the invention are used in a PCR it is convenient to detect the product by gel electrophoresis and ethidium bromide staining. As an alternative, it is convenient to use a labelled
25 oligonucleotide capable of hybridising to the amplified DNA as a probe. When the amplification is by PCR the oligonucleotide probe hybridises to the interprimer sequence as defined by the two primers. The oligonucleotide probe is preferably between 10 and 50 nucleotides long,

more preferably between 15 and 30 nucleotides long. It may be longer than the amplified DNA or include one or both of the primers, but in this case, the hybridisation conditions should be such that the probe should not hybridise to the primers alone, but only to an amplified product that also 5 contains interprimer sequence that is capable of hybridising to the probe.

The probe may be labelled with a radionuclide such as ^{32}P , ^{33}P and ^{35}S using standard techniques, or may be labelled with a fluorescent dye. When the oligonucleotide probe is fluorescently labelled, the amplified DNA product may be detected in solution (see for example Balaguer *et al* (1991). 10 "Quantification of DNA sequences obtained by polymerase chain reaction using a bioluminescence adsorbent" *Anal. Biochem.* 195, 105-110 and Dilesare *et al* (1993) "A high-sensitivity electrochemiluminescence-based detection system for automated PCR product quantitation" *BioTechniques* 15, 152-157.

15 PCR products can also be detected using a probe which may have a fluorophore-quencher pair or may be attached to a solid support or may have a biotin tag or they may be detected using a combination of a capture probe and a detector probe.

Fluorophore-quencher pairs are particularly suited to quantitative 20 measurements of PCR reactions (eg RT-PCR). Fluorescence polarisation using a suitable probe may also be used to detect PCR products.

The invention also includes a vector comprising the CRCV or CRCV-like polynucleotide of the fourth aspect of the invention.

Typical prokaryotic vector plasmids are: pUC18, pUC19, pBR322 and 25 pBR329 available from Biorad Laboratories (Richmond, CA, USA); pTrc99A, pKK223-3, pKK233-3, pDR540 and pRIT5 available from

Pharmacia (Piscataway, NJ, USA); pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16A, pNH18A, pNH46A available from Stratagene Cloning Systems (La Jolla, CA 92037, USA).

A typical mammalian cell vector plasmid is pSVL available from Pharmacia (Piscataway, NJ, USA). This vector uses the SV40 late promoter to drive expression of cloned genes, the highest level of expression being found in T antigen-producing cells, such as COS-1 cells. An example of an inducible mammalian expression vector is pMSG, also available from Pharmacia (Piscataway, NJ, USA). This vector uses the glucocorticoid-inducible promoter of the mouse mammary tumour virus long terminal repeat to drive expression of the cloned gene.

Useful yeast plasmid vectors are pRS403-406 and pRS413-416 and are generally available from Stratagene Cloning Systems (La Jolla, CA 92037, USA). Plasmids pRS403, pRS404, pRS405 and pRS406 are Yeast Integrating plasmids (YIps) and incorporate the yeast selectable markers *HIS3*, *TRP1*, *LEU2* and *URA3*. Plasmids pRS413-416 are Yeast Centromere plasmids (YCps).

Generally, the CRCV or CRCV-like polynucleotide of the invention is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression. It may be linked to the appropriate transcriptional and translational regulatory control nucleotide sequences recognised by the desired host prior to insertion into the vector, although such controls are generally available in the expression vector. Thus, the polynucleotide of the invention insert may be operatively linked to an appropriate promoter. Eukaryotic promoters include the CMV immediate early promoter, the HSV thymidine kinase promoter, the early and late SV40 promoters and the promoters of retroviral LTRs. Other suitable promoters will be known to the skilled artisan. The expression constructs desirably also

contain sites for transcription initiation and termination, and in the transcribed region, a ribosome binding site for translation (Hastings *et al*, International Patent No. WO 98/16643).

Methods well known to those skilled in the art can be used to construct expression vectors containing the coding sequence and, for example appropriate transcriptional or translational controls. One such method involves ligation via homopolymer tails. Homopolymer polydA (or polydC) tails are added to exposed 3' OH groups on the DNA fragment to be cloned by terminal deoxynucleotidyl transferases. The fragment is then capable of annealing to the polydT (or polydG) tails added to the ends of a linearised plasmid vector. Gaps left following annealing can be filled by DNA polymerase and the free ends joined by DNA ligase.

Another method involves ligation via cohesive ends. Compatible cohesive ends can be generated on the DNA fragment and vector by the action of suitable restriction enzymes. These ends will rapidly anneal through complementary base pairing and remaining nicks can be closed by the action of DNA ligase.

A further method uses synthetic molecules called linkers and adaptors. DNA fragments with blunt ends are generated by bacteriophage T4 DNA polymerase or *E.coli* DNA polymerase I which remove protruding 3' termini and fill in recessed 3' ends. Synthetic linkers, pieces of blunt-ended double-stranded DNA which contain recognition sequences for defined restriction enzymes, can be ligated to blunt-ended DNA fragments by T4 DNA ligase. They are subsequently digested with appropriate restriction enzymes to create cohesive ends and ligated to an expression vector with compatible termini. Adaptors are also chemically synthesised DNA fragments which contain one blunt end used for ligation but which also possess one preformed cohesive end.

Synthetic linkers containing a variety of restriction endonuclease sites are commercially available from a number of sources including International Biotechnologies Inc, New Haven, CN, USA.

A desirable way to modify the polynucleotide of the invention is to use the 5 polymerase chain reaction as disclosed by Saiki *et al* (1988) *Science* **239**, 487-491. In this method the DNA to be enzymatically amplified is flanked by two specific oligonucleotide primers which themselves become incorporated into the amplified DNA. The specific primers may contain restriction endonuclease recognition sites which can be used for cloning into expression 10 vectors using methods known in the art.

The invention also includes a host cell transformed with the vector comprising the CRCV or CRCV-like polynucleotide. The host cell can be either prokaryotic or eukaryotic. If the CRCV or CRCV-like polynucleotide, in the vector, is to be expressed as a glycoprotein, the host 15 cell is a eukaryotic host cell, and preferably a mammalian host cell.

Bacterial cells are preferred prokaryotic host cells and typically are a strain of *E. coli* such as, for example, the *E. coli* strains DH5 available from Bethesda Research Laboratories Inc., Bethesda, MD, USA, and RR1 available from the American Type Culture Collection (ATCC) of Rockville, 20 MD, USA (No ATCC 31343). Preferred eukaryotic host cells include yeast and mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human fibroblastic cell line. Yeast host cells include YPH499, YPH500 and YPH501 which are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Preferred 25 mammalian host cells include Chinese hamster ovary (CHO) cells available from the ATCC as CCL61, NIH Swiss mouse embryo cells NIH/3T3 available from the ATCC as CRL 1658, and monkey kidney-derived COS-1

cells available from the ATCC as CRL 1650. Preferred insect cells are Sf9 cells which can be transfected with baculovirus expression vectors.

Transformation of appropriate cell hosts with a vector is accomplished by well known methods that typically depend on the type of vector used. With regard to transformation of prokaryotic host cells, see, for example, Cohen *et al* (1972) *Proc. Natl. Acad. Sci. USA* **69**, 2110 and Sambrook *et al* (2001)-*Molecular Cloning, A Laboratory Manual*, 3rd Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. Transformation of yeast cells is described in Sherman *et al* (1986) *Methods In Yeast Genetics, A Laboratory Manual*, Cold Spring Harbor, NY. The method of Beggs (1978) *Nature* **275**, 104-109 is also useful. With regard to vertebrate cells, reagents useful in transfecting such cells, for example calcium phosphate and DEAE-dextran or liposome formulations, are available from Stratagene Cloning Systems, or Life Technologies Inc., Gaithersburg, MD 20877, USA.

Electroporation is also useful for transforming cells and is well known in the art for transforming yeast cell, bacterial cells and vertebrate cells.

For example, many bacterial species may be transformed by the methods described in Luchansky *et al* (1988) *Mol. Microbiol.* **2**, 637-646 incorporated herein by reference. The greatest number of transformants is consistently recovered following electroporation of the DNA-cell mixture suspended in 2.5x PEB using 6250V per cm at 25μFD.

Methods for transformation of yeast by electroporation are disclosed in Becker & Guarente (1990) *Methods Enzymol.* **194**, 182.

Physical methods may be used for introducing DNA into animal and plant cells. For example, microinjection uses a very fine pipette to inject DNA molecules directly into the nucleus of the cells to be transformed. Another

example involves bombardment of the cells with high-velocity microparticles, usually particles of gold or tungsten that have been coated with DNA.

Successfully transformed cells, ie cells that contain a CRCV or CRCV-like 5 DNA construct, can be identified by well known techniques. For example, one selection technique involves incorporating into the expression vector a DNA sequence (marker) that codes for a selectable trait in the transformed cell. These markers include dihydrofolate reductase, G418 or neomycin resistance for eukaryotic cell culture, and tetracyclin, kanamycin or ampicillin 10 resistance genes for culturing in *E.coli* and other bacteria. Alternatively, the gene for the selectable trait can be on another vector, which is used to co-transform the desired host cell.

The marker gene can be used to identify transformants but it is desirable to determine which of the cells contain recombinant DNA molecules and which 15 contain self-ligated vector molecules. This can be achieved by using a cloning vector where insertion of a DNA fragment destroys the integrity of one of the genes present on the molecule. Recombinants can therefore be identified because of loss of function of that gene.

Another method of identifying successfully transformed cells involves 20 growing the cells resulting from the introduction of an expression construct of the present invention to produce the CRCV or CRCV-like S, pol or HE protein. Cells can be harvested and lysed and their DNA content examined for the presence of the DNA using a method such as that described by Southern (1975) *J. Mol. Biol.* **98**, 503 or Berent *et al* (1985) *Biotech.* **3**, 208. 25 Alternatively, the presence of the protein in the supernatant can be detected using antibodies as described below.

In addition to directly assaying for the presence of recombinant DNA, successful transformation can be confirmed by well known immunological methods when the recombinant DNA is capable of directing the expression of the protein. For example, cells successfully transformed with an expression 5 vector produce proteins displaying appropriate antigenicity. Samples of cells suspected of being transformed are harvested and assayed for the protein using suitable antibodies.

Thus, in addition to the transformed host cells themselves, the present invention also contemplates a culture of those cells, preferably a monoclonal 10 (clonally homogeneous) culture, or a culture derived from a monoclonal culture, in a nutrient medium.

Host cells that have been transformed by the recombinant CRCV or CRCV-like polynucleotide, typically in a vector as described above, are then cultured for a sufficient time and under appropriate conditions known to those skilled 15 in the art in view of the teachings disclosed herein to permit the expression of the CRCV or CRCV-like protein encoded by the CRCV or CRCV-like polynucleotide, which can then be recovered.

The CRCV or CRCV-like protein can be recovered and purified from recombinant cell cultures by well-known methods including ammonium 20 sulphate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography ("HPLC") is employed for purification.

25 For example, for expression in a baculovirus system, recombinant DNA encoding the CRCV spike gene may be cloned into a suitable transfer vector such as pMelBac (Invitrogen). Co-transfection with baculovirus DNA (eg

Bac-N-Blue/Invitrogen) results in a recombinant baculovirus encoding the spike gene. Infection of a suitable insect cell line (e.g. Sf9, Sf21, High Five/Invitrogen) at an appropriate multiplicity of infection leads to expression of the recombinant spike protein. Protein expression is 5 confirmed by western blotting or ELISA using appropriate reagents (e.g. convalescent canine serum or other virus specific antiserum).

The invention thus includes a method of obtaining a CRCV or CRCV-like protein encoded by the CRCV or CRCV-like polynucleotide of the present invention. The method comprises culturing the host cell comprising the 10 CRCV or CRCV-like polynucleotide, typically in a vector; expressing the protein in the host cell, and purifying the protein. The invention further includes the protein obtainable by this method.

The invention thus also includes a method of obtaining a glycosylated CRCV or CRCV-like protein, typically an S protein, encoded by the CRCV 15 or CRCV-like polynucleotide of the present invention. The method comprises culturing a eukaryotic, or more preferably mammalian, host cell comprising the CRCV or CRCV-like polynucleotide, typically in a vector; expressing the protein in the host cell; and purifying the glycosylated protein. The invention further includes the glycosylated protein obtainable 20 by this method.

In a fifth aspect, the invention provides a method of making an anti-CRCV antibody comprising raising an immune response to a CRCV or CRCV-like S protein of the invention as described above in the first aspect of the invention in an animal, and preparing an antibody from the animal or from 25 an immortal cell derived therefrom. Alternatively, the method may comprise selecting an antibody from an antibody-display library using a CRCV or CRCV-like S protein of the invention as described above in the first aspect of the invention.

Methods and techniques for producing a monoclonal antibody are well known to a person of skill in the art, for example those disclosed in "Monoclonal Antibodies: A manual of techniques", H Zola (CRC Press, 1988) and in "Monoclonal Hybridoma Antibodies: Techniques and Applications", J G R Hurrell (CRC Press, 1982), incorporated herein by reference.

Optionally, the method further comprises determining whether the antibody thus obtained has greater affinity for the CRCV S protein than for the BCV S protein, and preferably also whether the antibody has a greater affinity for 10 the CRCV S protein than for the HCV and HEV S proteins. Methods for determining the relative affinity of antibodies for antigens are known in the art.

The invention also includes an anti-CRCV antibody obtainable by the method of the fifth aspect of the invention, that has greater affinity for the 15 CRCV S protein than for the BCV S protein. Preferably, the antibody also has a greater affinity for the CRCV S protein than for the HCV and HEV S proteins.

The invention also includes a method of making an anti-CRCV antibody comprising raising an immune response to a CRCV or CRCV-like HE 20 protein of the invention as described above in the third aspect of the invention in an animal, and preparing an antibody from the animal or from an immortal cell derived therefrom. Alternatively, the method may comprise selecting an antibody from an antibody-display library using a CRCV or CRCV-like HE protein of the invention as described above in the 25 third aspect of the invention.

Optionally, the method further comprises determining whether the antibody thus obtained has greater affinity for the CRCV HE protein than for the

BCV HE protein, and preferably also whether the antibody has a greater affinity for the CRCV HE protein than for the HCV and HEV HE proteins. Methods for determining the relative affinity of antibodies for antigens are known in the art.

5 The invention also includes an anti-CRCV antibody obtainable by the method of the fifth aspect of the invention, that has greater affinity for the CRCV HE protein than for the BCV HE protein. Preferably, the antibody also has a greater affinity for the CRCV HE protein than for the HCV and HEV HE proteins.

10 Preferably, the antibody is a monoclonal antibody. However, the invention includes a monospecific anti-CRCV antibody. The antibody may be an antibody fragment, as described below.

15 The monoclonal or monospecific antibody may be a chimaeric antibody, as discussed by Neuberger *et al* (1988, *8th International Biotechnology Symposium* Part 2, 792-799). The monoclonal or monospecific antibody may also be a “caninised” antibody, for example by inserting the CDR regions of mouse antibodies into the framework of canine antibodies.

20 The invention also includes anti-CRCV antibody fragments. The variable heavy (V_H) and variable light (V_L) domains of antibodies are involved in antigen recognition, a fact first recognised by early protease digestion experiments. Further confirmation was found by “humanisation” of rodent antibodies, in which variable domains of rodent origin may be fused to constant domains of human origin such that the resultant antibody retains the antigenic specificity of the rodent parented antibody (Morrison *et al* (1984) 25 *Proc. Natl. Acad. Sci. USA* **81**, 6851-6855).

That antigenic specificity is conferred by variable domains and is independent of the constant domains is known from experiments involving the bacterial expression of antibody fragments, all containing one or more variable domains.

5 These molecules include Fab-like molecules (Better *et al* (1988) *Science* **240**, 1041); Fv molecules (Skerra *et al* (1988) *Science* **240**, 1038); single-chain Fv (ScFv) molecules where the V_H and V_L partner domains are linked via a flexible oligopeptide (Bird *et al* (1988) *Science* **242**, 423; Huston *et al* (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5879) and single domain antibodies (dAbs) comprising isolated V domains (Ward *et al* (1989) *Nature* **341**, 544). A general review of the techniques involved in the synthesis of antibody fragments which retain their specific binding sites is to be found in Winter & Milstein (1991) *Nature* **349**, 293-299.

10

15 By "ScFv molecules" we mean molecules wherein the V_H and V_L partner domains are linked via a flexible oligopeptide.

The advantages of antibody fragments, rather than whole antibodies, are several-fold. The smaller size of the fragments may lead to improved pharmacological properties, such as better penetration of solid tissue. Effector functions of whole antibodies, such as complement binding, are removed.
20 Fab, Fv, ScFv and dAb antibody fragments can all be expressed in and secreted from *E. coli*, thus allowing the facile production of large amounts of the fragments.

Whole antibodies, and F(ab')₂ fragments are "bivalent". By "bivalent" we mean that the antibodies and F(ab')₂ fragments have two antigen combining sites. In contrast, Fab, Fv, ScFv and dAb fragments are monovalent, having only one antigen combining sites.
25

In a sixth aspect, the invention provides a method of determining whether a dog has been exposed to CRCV. The method comprises obtaining a suitable sample from the dog, and identifying CRCV or an anti-CRCV antibody in the sample. The method may be used as an aid in the diagnosis
5 of whether a dog has CIRD.

The invention includes a method of detecting, in a sample obtained from a dog, past exposure of the dog to CRCV, the method comprising obtaining a suitable sample from the dog, and identifying anti-CRCV antibodies in the sample.

10 In one preferred embodiment, the suitable sample can be any antibody containing sample such as serum, saliva, tracheal wash or bronchiolar lavage.

Preferably, the anti-CRCV antibody can be detected using a BCV, HCV, HEV or CRCV antigen, more preferably, using a BCV or CRCV antigen.

15 More preferably, identifying an anti-CRCV antibody in the sample comprises identifying an antibody that selectively binds to an S protein whose amino acid sequence is at least 75% identical with the amino acid sequence of the CRCV S protein (Figure 4 and SEQ ID NO: 4); an S protein whose amino acid sequence is at least 75% identical with the amino acid
20 sequence of the BCV S protein (Genbank Accession No. AF058942); HCV S protein (Genbank Accession No. L14643); to a coronavirus having an S protein at least 75% identical with BCV S protein (Genbank Accession No. AF058942), or a fragment thereof; or to a coronavirus having an S protein at least 75% identical with the CRCV S protein, or a fragment thereof.

25 More preferably, identifying an antibody that selectively binds to an S protein whose amino acid sequence is at least 75% identical with the amino

acid sequence of the BCV S protein, comprises identifying an antibody that selectively binds to an S protein whose amino acid sequence is at least 80% identical, or at least 85% identical, or at least 90% identical, or at least 95% identical with the amino acid sequence of the BCV S protein (Genbank Accession No. AF058942) or a fragment thereof.

More preferably, identifying an anti-CRCV antibody in the sample comprises identifying an antibody that selectively binds to the BCV S protein (Genbank Accession No. AF058942).

Even more preferably, identifying an antibody that selectively binds to an S protein whose amino acid sequence is at least 75% identical with the amino acid sequence of the CRCV S protein, comprises identifying an antibody that selectively binds to an S protein whose amino acid sequence is at least 80% identical, or at least 85% identical, or at least 90% identical, or at least 95% identical with the amino acid sequence of the CRCV S protein (Figure 4 and SEQ ID NO: 4) or a fragment thereof.

Yet more preferably, identifying an anti-CRCV antibody in the sample comprises identifying an antibody that selectively binds to a CRCV or CRCV-like S protein as defined in the first aspect of the invention.

Most preferably, identifying an anti-CRCV antibody in the sample comprises identifying an antibody that selectively binds to the CRCV S protein as listed in Figure 4 (SEQ ID NO: 4), or a fragment thereof.

Similarly, identifying an anti-CRCV antibody in the sample may comprise identifying an antibody that selectively binds to an HE protein whose amino acid sequence is at least 90% identical with the partial amino acid sequence of the CRCV HE protein (Figure 14 and SEQ ID NO: 22); to an HE protein whose amino acid sequence is at least 90% identical with the amino acid

sequence of the BCV HE protein (Genbank Accession No. AF058942) or the HECV HE protein (Genbank Accession No. L07747); to a coronavirus having an S protein at least 90% identical with BCV HE protein (Genbank Accession No. AF058942), or a fragment thereof; or to a coronavirus
5 having an HE protein at least 90% identical with the CRCV HE protein, or a fragment thereof.

More preferably, identifying an antibody that selectively binds to an HE protein whose amino acid sequence is at least 90% identical with the amino acid sequence of the BCV HE protein, comprises identifying an antibody
10 that selectively binds to an HE protein whose amino acid sequence is at least 91% identical, or at least 92% identical, or at least 93% identical, or at least 94% identical, or at least 95% identical, or at least 96% identical, or at least 97% identical, or at least 98% identical, or at least 99% identical with the amino acid sequence of the BCV HE protein (Genbank Accession No.
15 AF058942) or a fragment thereof.

More preferably, identifying an anti-CRCV antibody in the sample comprises identifying an antibody that selectively binds to the BCV HE protein (Genbank Accession No. AF058942).

Even more preferably, identifying an antibody that selectively binds to an
20 HE protein whose amino acid sequence is at least 90% identical with the partial amino acid sequence of the CRCV HE protein, comprises identifying an antibody that selectively binds to an HE protein whose partial amino acid sequence is at least 91% identical, or at least 92% identical, or at least 93% identical, or at least 94% identical, or at least 95% identical, or at least 96% identical, or at least 97% identical, or at least 98% identical, or at least 99% identical with the partial amino acid sequence of the CRCV HE protein
25 (Figure 13) or a fragment thereof.

Yet more preferably, identifying an anti-CRCV antibody in the sample comprises identifying an antibody that selectively binds to a CRCV or CRCV-like HE protein as defined in the third aspect of the invention.

Most preferably, identifying an anti-CRCV antibody in the sample
5 comprises identifying an antibody that selectively binds to the CRCV HE protein whose partial amino acid sequence is listed in Figure 14 (SEQ ID NO: 22), or a fragment thereof.

The invention includes a method of detecting CRCV in a sample obtained from a dog, the method comprising obtaining a suitable sample from the
10 dog, and identifying CRCV in the sample.

It is appreciated that there may be some naturally occurring sequence variation between different isolates of CRCV. The invention thus includes identifying CRCV isolates whose S, pol and HE genes and proteins have some sequence variation from the sequences provided in Figures 1 to 4 and
15 13 and 14. It is appreciated, however, that the same methods will be used to detect the variant isolates of CRCV, as well as the isolate characterised by the sequences listed in Figures 1 to 4 and 13 and 14.

In a preferred embodiment, the suitable sample can be a lung wash, tracheal wash, tonsillar swab or a biopsy or post-mortem sample from the respiratory
20 tract of the dog.

Preferably, in this embodiment, identifying CRCV comprises identifying a nucleic acid component of CRCV.

Typically, this will be performed by extracting RNA from the sample, and obtaining cDNA therefrom, for example as is described in Example 1.
25 Thereafter, a CRCV nucleic acid component is identified in the cDNA, for example using techniques involving high stringency hybridisation, specific

amplification, and nucleotide sequencing, as are well known to a person of skill in the art (Sambrook *et al* (2001) *supra*).

Preferably, identifying CRCV comprises identifying a polynucleotide that hybridises at high stringency to the BCV genome, such as the LY138 strain genome (Genbank Accession No. AF058942) or a portion thereof.

Further preferably, identifying CRCV comprises identifying a polynucleotide that hybridises at high stringency to the CRCV S, pol or HE polynucleotides (Figures 1, 3 and 13) or a portion thereof.

By "hybridising at high stringency" is meant that the polynucleotide and the nucleic acid to which it hybridises have sufficient nucleotide sequence similarity that they can hybridise under highly stringent conditions. As is well known in the art, the stringency of nucleic acid hybridisation depends on factors such as length of nucleic acid over which hybridisation occurs, degree of identity of the hybridising sequences and on factors such as temperature, ionic strength and CG or AT content of the sequence.

Nucleic acids which can hybridise at high stringency to the CRCV cDNA molecule include nucleic acids which have >90% sequence identity, preferably those with >95% or >96% or >97% or >98, more preferably those with >99% sequence identity, over at least a portion of the CRCV cDNA.

Typical highly stringent hybridisation conditions which lead to selective hybridisation are known in the art, for example those described in Sambrook *et al* 2001 (*supra*), incorporated herein by reference.

An example of a typical hybridisation solution when a nucleic acid is immobilised on a nylon membrane and the probe nucleic acid is ≥ 500 bases is:

6 x SSC (saline sodium citrate)

0.5% sodium dodecyl sulphate (SDS)

100 µg/ml denatured, fragmented salmon sperm DNA

The hybridisation is performed at 68°C. The nylon membrane, with the

5 nucleic acid immobilised, may be washed at 68°C in 0.1 x SSC.

20 x SSC may be prepared in the following way. Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 ml of H₂O. Adjust the pH to 7.0 with a few drops of a 10 N solution of NaOH. Adjust the volume to 1 litre with H₂O. Dispense into aliquots. Sterilise by autoclaving.

10 An example of a typical hybridisation solution when a nucleic acid is immobilised on a nylon membrane and the probe is an oligonucleotide of between 15 and 50 bases is:

3.0 M trimethylammonium chloride (TMACl)

0.01 M sodium phosphate (pH 6.8)

15 1 mm EDTA (pH 7.6)

0.5% SDS

100 µg/ml denatured, fragmented salmon sperm DNA

0.1% non-fat dried milk

The optimal temperature for hybridisation is usually chosen to be 5°C
20 below the T_i for the given chain length. T_i is the irreversible melting temperature of the hybrid formed between the probe and its target sequence. Jacobs *et al* (1988) *Nucl. Acids Res.* **16**, 4637 discusses the determination of T_is. The recommended hybridization temperature for 17-mers in 3M

TMACl is 48-50°C; for 19-mers, it is 55-57°C; and for 20-mers, it is 58-66°C.

Preferably, identifying CRCV comprises using a polynucleotide having at least 80%, or at least 85%, or at least 90%, or at least 95% identity with a portion of the BCV genome (Genbank Accession No. AF058942).

More preferably, identifying CRCV comprises using a polynucleotide having at least 80%, or at least 85%, or at least 90%, or at least 95% identity with a portion of the CRCV S polynucleotide (Figure 3), or having at least 90%, or at least 95% identity with a portion of the CRCV polynucleotide (Figure 1), or having at least 90%, or at least 95% identity with a portion of the CRCV HE polynucleotide (Figure 13).

More preferably, identifying CRCV comprises identifying a CRCV polynucleotide as defined above with respect to the fourth aspect of the invention.

Most preferably, identifying CRCV comprises identifying a CRCV polynucleotide comprising or consisting of a sequence listed in Figure 1 or Figure 3 or Figure 13, or a fragment thereof.

In another preferred embodiment, identifying CRCV comprises identifying a protein component of CRCV.

Preferably, identifying a protein component of CRCV comprises identifying a CRCV protein as defined above in the first or second or third aspects of the invention.

Most preferably, identifying a protein component of CRCV comprises identifying a CRCV protein comprising or consisting of the amino acid sequence listed in Figure 2 or Figure 4 or Figure 14, or a fragment thereof.

Assaying a protein component of CRCV in a biological sample can occur using any art-known method. Preferred for assaying CRCV protein levels in a biological sample are antibody-based techniques.

Preferably, identifying a protein component of CRCV comprises using an antibody reactive with CRCV.

More preferably, the antibody reactive with CRCV is an anti-BCV antibody, an anti-HCV antibody, an anti-HEV antibody, or an anti-CRCV antibody obtainable or obtained by the methods of the fifth aspect of the invention.

For example, CRCV protein expression can be studied with classical immunohistological methods. In these, the specific recognition is provided by the primary antibody (polyclonal or monoclonal) but the secondary detection system can utilise fluorescent, enzyme, or other conjugated secondary antibodies. As a result, an immunohistological staining of tissue section for pathological examination is obtained. Tissues can also be extracted, e.g., with urea and neutral detergent, for the liberation of CRCV protein for Western-blot or dot/slot assay (Jalkanen, M., *et al*, *J. Cell. Biol.* 101:976-985 (1985); Jalkanen, M., *et al*, *J. Cell. Biol.* 105:3087-3096 (1987)). In this technique, which is based on the use of cationic solid phases, quantitation of CRCV protein can be accomplished using isolated CRCV protein as a standard. This technique can also be applied to body fluid samples.

Other antibody-based methods useful for detecting CRCV protein expression include immunoassays, such as the enzyme linked immunosorbent assay (ELISA) and the radioimmunoassay (RIA). For example, a CRCV reactive monoclonal antibody can be used both as an immunoadsorbent and as an enzyme-labeled probe to detect and quantify

the CRCV protein. The amount of CRCV protein present in the sample can be calculated by reference to the amount present in a standard preparation using a linear regression computer algorithm. Such an ELISA for detecting a tumour antigen is described in Iacobelli *et al*, *Breast Cancer Research and Treatment* 11: 19-30 (1988). In another ELISA assay, two distinct specific monoclonal antibodies can be used to detect CRCV protein in a body fluid. In this assay, one of the antibodies is used as the immunoadsorbent and the other as the enzyme-labeled probe.

The above techniques may be conducted essentially as a "one-step" or "two-step" assay. The "one-step" assay involves contacting CRCV protein with immobilized antibody and, without washing, contacting the mixture with the labeled antibody. The "two-step" assay involves washing before contacting the mixture with the labeled antibody. Other conventional methods may also be employed as suitable. It is usually desirable to immobilize one component of the assay system on a support, thereby allowing other components of the system to be brought into contact with the component and readily removed from the sample.

Suitable enzyme labels include, for example, those from the oxidase group, which catalyze the production of hydrogen peroxide by reacting with substrate. Glucose oxidase is particularly preferred as it has good stability and its substrate (glucose) is readily available. Activity of an oxidase label may be assayed by measuring the concentration of hydrogen peroxide formed by the enzyme-labeled antibody/substrate reaction. Besides enzymes, other suitable labels include radioisotopes, such as iodine (¹²⁵I, ¹²¹I), carbon (¹⁴C), sulfur (³⁵S), tritium (³H), indium (¹¹²In), and technetium (^{99m}Tc), and fluorescent labels, such as fluorescein and rhodamine, and biotin.

In a seventh aspect, the invention provides an immunosorbent assay for detecting anti-CRCV S or HE antibodies. The assay comprises a solid phase coated with a CRCV or CRCV-like S or HE protein, or coated with both CRCV or CRCV-like S and HE proteins as defined in the first and 5 third aspects of the invention, or obtainable using the methods of the fourth aspect of the invention, or an antigenic fragment thereof, wherein anti-CRCV S or HE antibodies in a sample exposed to the solid phase will bind to the protein; and a detectable label conjugate which will bind to the anti-CRCV antibodies bound to the solid phase.

10 It is appreciated that an antigenic fragment of the CRCV or CRCV-like S protein that coats the solid phase is of sufficient size to be bound by an anti-CRCV S antibody, and which comprises at least one of the amino acids specific for CRCV S protein as listed in Table 1.

15 It is also appreciated that an antigenic fragment of the CRCV or CRCV-like HE protein that coats the solid phase is of sufficient size to be bound by an anti-CRCV HE antibody, and which comprises at least one of the three amino acids specific for CRCV HE protein as defined above.

20 Preferably, the CRCV or CRCV-like S or HE protein, or antigenic fragment thereof, that coats the solid phase is at least 10 amino acids in length. More preferably, the CRCV or CRCV-like S protein, or antigenic fragment thereof, is at least 20, or at least 30, or at least 40, or at least 50, or at least 100, or at least 200, or at least 300, or at least 400 amino acids in length. The CRCV or CRCV-like S protein may be at least 500, or at least 600, or at least 700, or at least 800, or at least 900, or at least 1,000 amino acids in 25 length.

Preferably, the CRCV or CRCV-like S protein, or antigenic fragment thereof, that coats the solid phase is less than about 1200 amino acids in

length. More preferably, the CRCV or CRCV-like S protein, or antigenic fragment thereof, is less than about 1,100, or less than about 1,000, or less than about 900, or less than about 800, or less than about 700, or less than about 600, or less than about 500 amino acids in length. The CRCV or
5 CRCV-like S or HE protein may be less than about 400, or less than about 300, or less than about 200, or less than about 100, or less than about 50 amino acids in length.

Preferably, the solid phase is a microtitre well.

Further preferably, the conjugate comprises anti-dog antibody.

10 Preferably, the conjugate comprises an enzyme, for example horseradish peroxidase. Further preferably, the immunosorbent assay also comprises a substrate for the enzyme.

Further details of suitable immunosorbent assays and ELISAs are provided above.

15 The invention includes a kit of parts which include the components of the immunosorbent assay. The kit of parts may thus include a solid phase such as a microtitre plate, CRCV or CRCV-like S or HE protein or both for coating the solid phase, a detectable label conjugate, such as an anti-dog antibody, which will bind to anti-CRCV antibodies bound to the solid
20 phase. If the detectable label conjugate is an enzyme, the kit of parts may also include a substrate for the enzyme. The kit may also include a positive control sample that contains an anti-CRCV S or HE protein antibody, such as those described with reference to the fifth aspect of the invention, and a negative control sample.

25 The invention thus includes a solid substrate with a CRCV or CRCV-like S or HE protein as defined in the first and third aspects of the invention, or

obtainable using the methods of the fourth aspect of the invention, or an antigenic fragment thereof, attached thereto, for capturing anti-CRCV S or HE antibodies or both from a liquid sample, wherein anti-CRCV S or HE antibodies in a sample exposed to the solid substrate will bind to the S or
5 HE protein.

Typically, protein is coated on microtitre plates overnight at 4°C to 37°C, depending on the stability of the antigen. Unbound protein is washed off with a wash buffer such as phosphate buffered saline or Tris buffered saline. Serum or other samples are incubated on the plate, typically at 37°C for
10 between 1 and several hours. Unbound material is washed off, the plates are incubated with enzyme-labelled (e.g. horseradish peroxidase) antibody, such as anti-canine IgG or IgM for serum samples, or anti-canine IgA for lung washes, for 1 to several hours at 37°C. Unbound antibody is washed off and plates are incubated with a substrate such as OPD for about 10 min,
15 and the optical density measured in a photometer.

Preferably, the solid substrate is a microtitre well.

In an eighth aspect, the invention provides a vaccine composition for vaccinating dogs comprising (i) a coronavirus having an S protein with at least 75% amino acid identity with CRCV S protein, or (ii) a coronavirus having an S protein with at least 75% amino acid identity with BCV S protein, or (iii) a coronavirus having an HE protein with at least 90% amino acid identity with CRCV HE protein, or (iv) a coronavirus having an HE protein with at least 90% amino acid identity with BCV HE protein, or (v) a coronavirus protein having at least 75% amino acid identity with a CRCV protein or an immunogenic fragment thereof, or (vi) a coronavirus protein having at least 75% amino acid identity with a BCV protein or an immunogenic fragment thereof, or (vii) a nucleic acid encoding said coronaviral protein or immunogenic fraction thereof.

Preferably, the vaccine is packaged and presented for use in dogs.

When the vaccine comprises a coronavirus protein, or an immunogenic fragment thereof, the protein preferably has at least 80%, or at least 85%, or at least 90%, or at least 95% amino acid identity with the corresponding portion of a BCV or CRCV protein.

Preferably, the coronavirus protein is a BCV, HCV, HEV or CRCV protein, or a modification thereof.

Typical protein modifications include amino acid substitutions to improve the antigenicity of the vaccine. BCV, HCV and HEV proteins may be modified to be more like a CRCV protein. For example, the spike protein of BCV, HCV or HEV may be modified to include a CRCV amino acid at any of differences shown in the comparison in Figure 10, or listed in Table 1. Additionally or alternatively, the HE protein of BCV, HCV or HEV may be modified to include a CRCV amino acid at any of the three CRCV-specific residues as defined above.

Proteins in which one or more of the amino acid residues are chemically modified, may be used providing that the function of the protein, namely the production of specific antibodies *in vivo*, remains substantially unchanged. It is appreciated that synthesised proteins may be suitably modified before or after their synthesised. Such modifications include forming salts with acids or bases, especially physiologically acceptable organic or inorganic acids and bases, forming an ester or amide of a terminal carboxyl group, and attaching amino acid protecting groups such as N-t-butoxycarbonyl. Such modifications may protect the peptide from *in vivo* metabolism.

The protein may be present as single copies or as multiples, for example tandem repeats. Such tandem or multiple repeats may be sufficiently

antigenic themselves to obviate the use of a carrier. It may be advantageous for the protein to be formed as a loop, with the N-terminal and C-terminal ends joined together, or to add one or more Cys residues to an end to increase antigenicity and/or to allow disulphide bonds to be formed. If the protein is 5 covalently linked to a carrier, preferably a polypeptide, then the arrangement is preferably such that the protein of the invention forms a loop.

According to current immunological theories, a carrier function should be present in any immunogenic formulation in order to stimulate, or enhance stimulation of, the immune system. It is thought that the best carriers embody 10 (or, together with the antigen, create) a T-cell epitope. The peptides may be associated, for example by cross-linking, with a separate carrier, such as serum albumins, myoglobins, bacterial toxoids and keyhole limpet haemocyanin. More recently developed carriers which induce T-cell help in 15 the immune response include the hepatitis-B core antigen (also called the nucleocapsid protein), presumed T-cell epitopes such as Thr-Ala-Ser-Gly-Val-Ala-Glu-Thr-Thr-Asn-Cys (SEQ ID NO: 52), beta-galactosidase and the 163-171 peptide of interleukin-1. The latter compound may variously be regarded 20 as a carrier or as an adjuvant or as both. Alternatively, several copies of the same or different proteins of the invention may be cross-linked to one another; in this situation there is no separate carrier as such, but a carrier function may be provided by such cross-linking. Suitable cross-linking agents include those listed as such in the Sigma and Pierce catalogues, for example glutaraldehyde, carbodiimide and succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, the latter agent exploiting the -SH group on the C-terminal 25 cysteine residue (if present).

If the protein is prepared by expression of a suitable nucleotide sequence in a suitable host, then it may be advantageous to express it as a fusion product

with a peptide sequence which acts as a carrier. Kabigen's "Ecosec" system is an example of such an arrangement.

It is appreciated that the coronavirus component of the vaccine may be linked to other antigens to provide a dual effect.

- 5 Preferably, the coronavirus protein in the vaccine composition is an S protein. More preferably, the S protein is a CRCV or CRCV-like S protein as defined above in the first aspect of the invention or obtainable by the methods of the fourth aspect of the invention, a BCV S protein, an HCV S protein, an HEV S protein, or an immunogenic fragment thereof.
- 10 Most preferably, the vaccine composition contains a CRCV S protein that comprises or consists of the amino acid sequence listed in Figure 4, or an immunogenic fragment thereof having at least 97% identity with the sequence listed in Figure 4. Preferably, the variant has at least at least 98%, or at least 99% amino acid sequence identity with the sequence listed in
- 15 Figure 4. More preferably the variant has at least 99.1%, or at least 99.2%, or at least 99.3%, or at least 99.4%, or at least 99.5%, or at least 99.6%, or at least 99.7%, or at least 99.8%, or at least 99.9% amino acid sequence identity with the sequence listed in Figure 4.

Additionally or alternatively, the vaccine composition may comprise

20 coronavirus proteins such as a hemagglutinin-esterase protein (HE) or an integral membrane protein (M), or the small membrane protein (E) (Lai MMC & Cavanagh D, (1997) "The molecular biology of coronaviruses" *Adv. Vir. Res.*, 48: 1-100).

In one embodiment, the HE, E or M proteins are BCV, HCV or HEV proteins. In another embodiment, the HE, E or M proteins are CRCV proteins.

Preferably, the HE protein is a CRCV or CRCV-like HE protein as defined above in the third aspect of the invention or obtainable by the methods of the fourth aspect of the invention, or an immunogenic fragment thereof.

More preferably, the vaccine composition contains a CRCV HE protein that
5 comprises or consists of the partial amino acid sequence listed in Figure 14, or an immunogenic fragment thereof having at least 97% identity with the sequence listed in Figure 14. Preferably, the variant has at least at least 98%, or at least 99% amino acid sequence identity with the sequence listed in Figure 14. More preferably the variant has at least 99.1%, or at least 99.2%, or at least 99.3%, or at least 99.4%, or at least 99.5%, or at least 10 99.6%, or at least 99.7%, or at least 99.8%, or at least 99.9% amino acid sequence identity with the sequence listed in Figure 14.

When the vaccine comprises a coronavirus, preferably the coronavirus comprises an S protein with at least 80%, or at least 85%, or at least 90%, or 15 at least 95% amino acid identity with the BCV S protein. More preferably, the coronavirus comprises an S protein with at least 80%, or at least 85%, or at least 90%, or at least 95% amino acid identity with the CRCV S protein.

Additionally or alternatively, when the vaccine comprises a coronavirus, preferably the coronavirus comprises an HE protein with at least 90% or at 20 least 95% amino acid identity with the BCV HE protein. More preferably, the coronavirus comprises an HE protein with at least 96%, or at least 97%, or at least 98%, or at least 99% amino acid identity with the CRCV HE protein.

In another preferred embodiment, the vaccine composition comprises a 25 virus selected from BCV, HCV, HEV and CRCV, or a modification thereof.

It is appreciated that dog vaccines effective against a canine virus may be derived from a non-canine virus. For example US Patent No. 5,750,112 to Gill, and assigned to Solvay Animal Health Inc, discloses a vaccine against enteric canine coronavirus containing inactivated feline enteric coronavirus.

5 The disclosure of US 5,750,112 is incorporated herein by reference.

In one preferred embodiment, the virus is an inactivated virus. Methods for inactivating viruses for use in vaccines are well known in the art. Suitable methods include chemical methods, such as the use of beta propio-lactone (BPL). Suitable inactivated bovine coronavirus vaccines may include 10 inactivated BCV which is a component of bovine vaccines such as “Rotovec Corona” from Schering-Plough (<http://www.ukvet.co.uk/rotovec/scour.htm>); “Lactovac” by Hoechst Roussel Vet Ltd, (Veterinary Formulary 5th Edition of the Veterinary Data Sheet Compendium); “First Defense” by Immuncell Corp, USA; “Scour 15 Bos 4” by Grand Laboraotries and “Scour Guard 3K” by Pfizer.

In an alternative embodiment, the virus is an attenuated virus. Methods for attenuating viruses for use in vaccines are well known in the art.

Preferably, the vaccine composition also comprises a pharmaceutically acceptable adjuvant.

20 Preferably, when the vaccine comprises a nucleic acid, the nucleic acid encoding the coronaviral protein or immunogenic fraction thereof, for use as a vaccine is a CRCV or CRCV-like S polynucleotide, or a CRCV or CRCV-like HE polynucleotide or both a CRCV or CRCV-like S and HE polynucleotide. More preferably, the nucleic acid comprises or consists of 25 the nucleotide sequence listed in Figure 3 or Figure 13, or fractions thereof.

For vaccine use, the CRCV or CRCV-like S or HE nucleic acid can be delivered in various replicating (e.g. recombinant adenovirus vaccine) or non-replicating (DNA vaccine) vectors.

In a preferred embodiment, the vaccine may contain recombinant CRCV or
5 CRCV-like S protein, as well as other immunogenic coronavirus proteins such as the HE protein.

As discussed above, several viral and bacterial agents are known to be associated with respiratory disease in dogs, including canine parainfluenza virus (CPIV), canine adenovirus type 2 (CAV-2), canine herpesvirus
10 (CHV), and *Bordetella bronchiseptica* (*B. bronchiseptica*).

In another preferred embodiment, the vaccine may contain recombinant CRCV or CRCV-like S or HE protein, as well as other pathogenic organisms involved in respiratory disease of dogs such as canine parainfluenzavirus, canine adenovirus type 2, the bacterium *Bordetella*
15 *bronchiseptica*, canine herpesvirus, human reovirus and mycoplasma species, or immunogenic proteins therefrom. Thus the vaccine may contain an agent capable of raising an immune response, such as the production of antibodies against CRCV, as well as against other pathogenic organisms involved in respiratory disease of dogs such as CPIV, CAV-2, *B. bronchiseptica* and CHV.
20

In an embodiment, as well as containing an agent capable of stimulating the production of antibodies against CRCV, such as a CRCV or CRCV-like S or HE protein, the vaccine composition further comprises any one or more of:

25 (a) an agent capable of raising an immune response in a dog against CPIV;

(b) an agent capable of raising an immune response in a dog against CAV-2;

(c) an agent capable of raising an immune response in a dog against CHV; and

5 (d) an agent capable of raising an immune response in a dog against *B. bronchiseptica*.

Thus the vaccine composition can optionally also comprise any two, or any three or all four of these additional agents (a), (b), (c) and (d).

10 Typically, an agent capable of raising an immune response in a dog against CPIV comprises inactivated or attenuated CPIV, or an immunogenic fragment thereof, or a nucleic acid encoding said immunogenic fraction.

Typically, an agent capable of raising an immune response in a dog against CAV-2 comprises inactivated or attenuated CAV-2, or an immunogenic fragment thereof, or a nucleic acid encoding said immunogenic fraction.

15 Canine adenovirus type 1 causes infectious hepatitis; canine adenovirus type 2 causes respiratory disease. It has been shown that CAV-1 provides cross-protection against CAV-2 and vice versa. The agent that raises an immune response in a dog against CAV-2 may therefore contain either CAV-1 or CAV-2, or an immunogenic fragment thereof. The vaccines listed below contain CAV-2 except for EURICAN DHPPi, which does not 20 specify the virus type used.

Suitable agents that raise an immune response in a dog against CPIV and CAV-2 are known to a person of skill in the art. For example, the following dog vaccines are licensed in the UK.

KAVAK DA₂PiP69 by Fort Dodge Animal Health is a live freeze dried vaccine containing attenuated strains of canine distemper virus, canine adenovirus type 2, canine parainfluenza type 2 and canine parvovirus grown in tissue culture.

5 KAVAK Parainfluenza by Fort Dodge Animal Health contains live freeze-dried vaccine derived from an attenuated strain of canine parainfluenza virus type 2 cultivated on an established homologous cell-line.

NOBIVAC DHPPi by Intervet UK Limited is a live attenuated freeze-dried, virus vaccine containing canine distemper virus, canine adenovirus type 2, 10 canine parvovirus and canine parainfluenza virus grown in cell line tissue culture.

NOBIVAC KC by Intervet UK Limited is a modified live freeze-dried vaccine containing *Bordetella bronchiseptica* strain B-C2 and canine parainfluenza virus strain Cornell (this is an intranasal vaccine).
15 Management authorisation number Vm 06376/4026.

EURICAN DHPPi by Merial Animal Health Ltd. is a combined live freeze-dried vaccine against canine distemper, infectious canine hepatitis, canine parvovirus and canine parainfluenza virus type 2.

VANGUARD 7 by Pfizer Ltd. contains live attenuated canine distemper virus (Snyder Hill strain), adenovirus (CAV-2 Manhattan strain), parainfluenza virus (NL-CPI-5 strain), canine parvovirus (NL-35-D) propagated in an established cell line, and an inactivated culture of *Leptospira canicola* and *Leptospira icterohaemorrhagiae*.
20

QUANTUM DOG 7 by Schering-Plough Animal Health contains canine distemper, adenovirus type 2, parvovirus, parainfluenza virus type 2 vaccine
25

(living) and inactivated *Leptospira canicola* and *Leptospira icteroohaemorrhagiae* vaccine.

CANIGEN DHPPi by Virbac Ltd. is a live attenuated, freeze-dried, virus vaccine containing canine distemper virus, canine adenovirus (CAV2),
5 canine parvovirus and canine parainfluenza virus grown in cell line tissue culture.

CANIGEN Ppi by Virbac Ltd. is a live attenuated, freeze-dried virus vaccine containing canine parvovirus and canine parainfluenza virus grown in cell line tissue culture.

10 Typically, an agent capable of raising an immune response in a dog against CHV comprises inactivated or attenuated CHV, or an immunogenic fragment thereof, or a nucleic acid encoding said immunogenic fraction.

Suitable agents that raise an immune response in a dog against CHV are known to a person of skill in the art. For example, EURICAN Herpes 205
15 by Merial is a purified sub-unit vaccine against canine herpesvirus which is indicated for the active immunisation of pregnant bitches to prevent mortality, clinical signs and lesions in puppies resulting from canine herpesvirus infections acquired in the first days of life. It is not licensed for the vaccination of adult dogs for the prevention of respiratory disease.

20 Typically, an agent capable of raising an immune response in a dog against *B. bronchiseptica* comprises inactivated or attenuated *B. bronchiseptica*, or an immunogenic fragment thereof, or a nucleic acid encoding said immunogenic fraction.

25 Suitable agents that raise an immune response in a dog against *B. bronchiseptica* are known to a person of skill in the art. For example, the following dog vaccines are licensed for use.

COUGHGUARD-B® by Pfizer Animal Health (U.S. Vet. Lic. No.: 189) contains an inactivated culture of *B. bronchiseptica*. It is for the immunisation of healthy dogs against disease caused by *B. bronchiseptica*, in particular kennel cough. COUGHGUARD-B® is prepared from a highly antigenic strain of *B. bronchiseptica* which has been inactivated and processed to be nontoxic when administered to dogs. The production method is reported to leave the immunogenic properties of *B. bronchiseptica* intact.

VANGUARD® 5/B by Pfizer Animal Health (U.S. Vet. Lic. No.: 189) contains attenuated strains of canine distemper virus (CDV), CAV-2, CPIV, and canine parvovirus (CPV) propagated on an established canine cell line. The CPV antigen was attenuated by low passage on the canine cell line and at that passage level has immunogenic properties capable of overriding maternal antibodies. The vaccine is packaged in lyophilised form with inert gas in place of vacuum. The bacterin component containing inactivated whole cultures of *B. bronchiseptica* which is supplied as diluent. The *B. bronchiseptica* component in VANGUARD® 5/B is prepared from a highly antigenic strain which has been inactivated and processed to be nontoxic when administered to dogs.

NASAGUARD-B™ by Pfizer Animal Health (U.S. Vet. Lic. No.: 112) is composed of an avirulent live culture of *B. bronchiseptica* bacteria.

PROGARD®-KC by Intervet is a modified live intranasal vaccine containing attenuated canine parainfluenza virus and *Bordetella bronchiseptica* avirulent live culture. PROGARD®-KC is presented in a desiccated form with sterile diluent provided for reconstitution. PROGARD®-KC is for vaccination of healthy, susceptible puppies and dogs for prevention of canine infectious tracheobronchitis ("kennel cough") due to canine parainfluenza virus and *B. bronchiseptica*.

PROGARD®-KC PLUS by Intervet contains live culture of avirulent strains of *B. bronchiseptica*, attenuated canine adenovirus type 2 and parainfluenza virus for intranasal administration. Vaccination with PROGARD®-KC Plus stimulates rapid, local immunity in the respiratory tract, thereby inhibiting infection at the port of entry as well as preventing clinical signs. In addition to local immunity, it also stimulates systemic immunity within three weeks of intranasal administration. The small volume (0.4 ml) and one nostril application of PROGARD®-KC Plus provide for ease in vaccination, particularly in small breeds and young puppies. PROGARD®-KC Plus is presented in a desiccated form with sterile diluent provided for reconstitution. PROGARD®-KC Plus is for vaccination of healthy dogs and puppies three weeks of age or older for prevention of canine infectious tracheobronchitis ("kennel cough") due to canine adenovirus type 2, parainfluenza virus and *B. bronchiseptica*.

Intrac by Intervet is a freeze dried modified live vaccine, containing *B. bronchiseptica* strain S 55, for intranasal administration. Product licence number PL 0201/4011

Nobivac KC, described above, also contains *B. bronchiseptica*.

Vaccination would be useful especially but not exclusively for dogs prior to entry into a boarding kennel or for the vaccination of dogs in breeding facilities.

A typical dose of a vaccine comprised of recombinant protein is about 5-10 µg. A typical dose of a vaccine comprised of inactivated virus is about 1-10 mg.

In a ninth aspect, the invention provides the use of (i) a coronavirus having an S protein with at least 75% amino acid identity with CRCV S protein, or

(ii) a coronavirus having an S protein with at least 75% amino acid identity with BCV S protein, or (iii) a coronavirus having an HE protein with at least 90% amino acid identity with CRCV HE protein, or (iv) a coronavirus having an HE protein with at least 90% amino acid identity with BCV HE protein, or (v) a coronavirus protein having at least 75% amino acid identity with a CRCV protein or an immunogenic fragment thereof, or (vi) a coronaviral protein having at least 75% amino acid identity with a BCV protein, or an immunogenic fragment thereof, or (vii) a nucleic acid encoding said coronaviral protein or immunogenic fraction thereof, in the preparation of a medicament for stimulating an immune response against CRCV in a dog.

The invention includes the use of (i) a coronavirus having an S protein with at least 75% amino acid identity with CRCV S protein, or (ii) a coronavirus having an S protein with at least 75% amino acid identity with BCV S protein, or (iii) a coronavirus having an HE protein with at least 90% amino acid identity with CRCV HE protein, or (iv) a coronavirus having an HE protein with at least 90% amino acid identity with BCV HE protein, or (v) a coronavirus protein having at least 75% amino acid identity with a CRCV protein or an immunogenic fragment thereof, or (vi) a coronaviral protein having at least 75% amino acid identity with a BCV protein, or an immunogenic fragment thereof, or (vii) a nucleic acid encoding said coronaviral protein or immunogenic fraction thereof, in the preparation of a medicament for prophylaxis of respiratory disease in a dog, typically CIRD.

When a coronavirus protein, or an immunogenic fragment thereof, is used in the preparation of the medicament, the protein preferably has at least 80%, or at least 85%, or at least 90%, or at least 95% amino acid identity with the corresponding portion of a BCV protein. Preferably the protein has

at least 80%, or at least 85%, or at least 90%, or at least 95% amino acid identity with the corresponding portion of a CRCV protein.

Preferably, the coronaviral protein used in the preparation of the medicament is a BCV, HCV, HEV or CRCV protein, or a modification thereof, as described above with reference to the eighth aspect of the invention.

More preferably, the coronaviral protein used in the preparation of the medicament is an S protein. Yet more preferably, the S protein comprises an CRCV or CRCV-like S protein as defined above in the first aspect of the invention or obtainable by the methods of the fourth aspect of the invention, a BCV S protein, an HCV S protein, or an immunogenic fragment thereof.

Most preferably, the coronaviral protein used in the preparation of the medicament comprises or consists of the amino acid sequence listed in Figure 4, or an immunogenic fragment thereof having at least 97% identity with the sequence listed in Figure 4. Preferably, the variant has at least 98%, or at least 99% amino acid sequence identity with the sequence listed in Figure 4. More preferably the variant has at least 99.1%, or at least 99.2%, or at least 99.3%, or at least 99.4%, or at least 99.5%, or at least 99.6%, or at least 99.7%, or at least 99.8%, or at least 99.9% amino acid sequence identity with the sequence listed in Figure 4.

Additionally or alternatively, the coronaviral protein used in the preparation of the medicament may comprise HE, E, M or N coronavirus proteins. In one embodiment, the HE, E, M or N proteins are BCV, HCV or HEV proteins. In another embodiment, the HE, E, M or N proteins are CRCV proteins.

Typically, the HE protein comprises an CRCV or CRCV-like HE protein as defined above in the third aspect of the invention or obtainable by the methods of the fourth aspect of the invention, a BCV HE protein, an HCV HE protein, or an immunogenic fragment thereof.

5 Preferably, the coronaviral HE protein used in the preparation of the medicament comprises or consists of the partial amino acid sequence listed in Figure 14, or an immunogenic fragment thereof having at least 97% identity with the sequence listed in Figure 14. Preferably, the variant has at least 98%, or at least 99% amino acid sequence identity with the sequence listed in Figure 14. More preferably the variant has at least 99.1%, or at least 99.2%, or at least 99.3%, or at least 99.4%, or at least 99.5%, or at least 99.6%, or at least 99.7%, or at least 99.8%, or at least 99.9% amino acid sequence identity with the partial sequence listed in Figure 14.

10

When a coronavirus is used in the preparation of the medicament, the 15 coronavirus preferably comprises an S protein with at least 80%, or at least 85%, or at least 90%, or at least 95% amino acid identity with the BCV S protein. More preferably the coronavirus comprises an S protein with at least 80%, or at least 85%, or at least 90%, or at least 95% amino acid identity with the CRCV S protein.

20 Additionally or alternatively, the coronavirus may comprise an HE protein with at least 90%, or at least 95% amino acid identity with the BCV HE protein. More preferably the coronavirus comprises an HE protein with at least 96%, or at least 97%, or at least 98%, or at least 99% amino acid identity with the CRCV HE protein.

25 In a tenth aspect, the invention provides a CRCV or CRCV-like S protein as defined above in the first aspect of the invention or obtainable by the

methods of the fourth aspect of the invention, for use in medicine. Typically, the S protein will be used in veterinary medicine.

The invention includes a CRCV or CRCV-like HE protein as defined above in the third aspect of the invention or obtainable by the methods of the 5 fourth aspect of the invention, for use in medicine. Typically, the HE protein will be used in veterinary medicine.

In an eleventh aspect, the invention provides a method of vaccinating a dog against CRCV, the method comprising administering to the dog a vaccine composition as described above in the ninth aspect of the invention.

10 Typically, the vaccine will be administered *via* the intramuscular, subcutaneous or intranasal routes

In another embodiment, a dog can passively acquire immunity against CRCV by being administered an antibody that reacts with CRCV. The antibody that reacts with CRCV may be an anti-BCV, anti-HCV antibody, 15 but is preferably an anti-CRCV antibody. Preferably, the antibody that reacts with CRCV is an anti-S protein antibody an anti-HE protein antibody. Most preferably, the antibody that reacts with CRCV is an anti-CRCV S or HE protein antibody as described in the fifth aspect of the invention.

20 In a twelfth aspect, the invention provides a method for combating the spread of CRCV between dogs comprising determining whether a dog is infected with CRCV according to the methods as described above in the sixth aspect of the invention, or using the immunosorbent assay or solid substrate as described above in the seventh aspect of the invention, and, if the dog is infected with CRCV, quarantining the dog.

25 By "quarantining" a dog we include the meaning of keeping the dog separate from all other dogs. We also include the meaning of keeping the

dog separate from dogs that have not been vaccinated against CRCV, which can be performed as described above. We also include the meaning of keeping the dog separate from dogs that have not been infected by CRCV, which can be determined as described above.

5 In a thirteenth aspect, the invention provides a method for combating the spread of CRCV between dogs comprising determining whether a dog is infected with CRCV according to the methods described above in the sixth aspect of the invention, or using the immunosorbent assay or solid substrate as described above in the seventh aspect of the invention, and, if the dog is
10 infected with CRCV, vaccinating other dogs that have been, are, or are likely to be in contact with the dog.

A fourteenth aspect of the invention provides a method for identifying a test vaccine capable of preventing or reducing the incidence of canine infectious respiratory disease (CIRD) in dogs. The method comprises (a) determining whether a dog has been exposed to CRCV, typically according to the methods described above in the sixth aspect of the invention or using the immunosorbent assay or solid substrate as described above in the seventh aspect of the invention, (b) if the dog has not been exposed to CRCV, administering the test vaccine to the dog, (c) inoculating the dog with CRCV, and (d) determining whether the dog develops CIRD. The absence of CIRD in step (d) indicates that the test vaccine is capable of preventing CIRD.
15
20

Typically, this method is performed on a set of dogs.

Preferably, the method involves the use of a set of control dog which are not administered the test vaccine in step (b). The significantly lower incidence of CIRD in the set of dogs that has been administered the test vaccine than
25

in the control set indicates that the test vaccine is capable of preventing or reducing the incidence of CIRD.

The invention also includes a vaccine identified by this method.

All of the documents referred to herein are incorporated herein, in their
5 entirety, by reference.

The invention will now be described in more detail with the aid of the following Figures and Examples.

Figure 1

Partial nucleotide sequence (250 residues) of the CRCV polymerase (pol)
10 cDNA (SEQ ID NO: 1).

Figure 2

Partial amino acid sequence (83 residues) of the CRCV pol protein (SEQ ID NO: 2), derived from the nucleotide sequence of Figure 1.

Figure 3

15 Nucleotide sequence (4092 residues) of the CRCV Spike (S) cDNA (SEQ ID NO: 3). The Y at position 3531 refers to either C or T.

Figure 4

Amino acid sequence (1363 residues) of the CRCV S protein (SEQ ID NO: 4), derived from the nucleotide sequence of Figure 3.

Figure 5

Consensus tree for cDNA sequences from a 250 nucleotide region of the polymerase gene of 12 coronaviruses. The sequence obtained from the canine respiratory coronavirus is designated T101. The numbers indicate 5 bootstrap values obtained by analysis of 100 data sets.

BCV: bovine coronavirus, CCV: canine coronavirus, FIPV: feline infectious peritonitis virus, HEV: hemagglutinating encephalomyelitis virus, IBV: infectious bronchitis virus, MHV: mouse hepatitis virus, OC43: human coronavirus strain OC43, SDAV: sialodacryoadenitis virus, TCV: turkey 10 coronavirus, TGEV: transmissible gastroenteritis virus, 229E: human coronavirus strain 229E, T101: canine respiratory coronavirus (PCR product from tracheal sample T101)

Figure 6

CLUSTAL X (1.8) multiple sequence alignment of the 250 nucleotide 15 partial sequence of the pol cDNA of CRCV (sample T101, SEQ ID NO: 1), BCV (SEQ ID NO: 5), HCV strain OC43 (SEQ ID NO: 6), HEV (SEQ ID NO: 7) and CCV (enteric CCV, SEQ ID NO: 8).

Figure 7

CLUSTAL X (1.8) multiple sequence alignment of the 83 amino acid 20 partial sequence of the pol protein of CRCV (protCRCVpol, SEQ ID NO: 2) with HCV (protHCVpoly, SEQ ID NO: 9), HEV (protHEVpoly, SEQ ID NO: 10), BCV (protBCVpoly, SEQ ID NO: 11) and CECV (enteric CCV, protCECVpol, SEQ ID NO: 12).

Figure 8

CLUSTAL X (1.8) sequence alignment of the nucleotide sequence of the CRCV spike cDNA (CRCVs^{pike}, SEQ ID NO: 3) and enteric CCV spike cDNA (CECVs^{pike}, SEQ ID NO: 13).

5 **Figure 9**

CLUSTAL X (1.8) multiple sequence alignment of the 4092 nucleotides of the CRCV spike cDNA (CRCVs^{pike}, SEQ ID NO: 3) sequence with BCV (BCVs^{pike}, SEQ ID NO: 14), HCV (HCVs^{pike}, SEQ ID NO: 15) and HEV (HEVs^{pike}, SEQ ID NO: 16) spike cDNAs. The Y at position 3531 in the
10 CRCV sequence refers to either C or T.

Figure 10

CLUSTAL X (1.8) multiple sequence alignment of the 1363 amino acid sequence of the CRCV spike protein (CRCVs^{pikepr}, SEQ ID NO: 4) with BCV (BCVs^{pikepro}, SEQ ID NO: 17), HCV (HCVs^{pikepro}, SEQ ID NO: 18), HEV (HEVs^{pikepro}, SEQ ID NO: 19) and enteric CCV (CECVs^{pikepr}, SEQ ID NO: 20) spike proteins.
15

Figure 11

RT-PCR using nested set of primers (Spike 1 and 2 (SEQ ID NOS: 34 and 35) followed by Spike 3 and 4 (SEQ ID NOS: 36 and 37)). BCV: Bovine
20 coronavirus positive control sample; A72: Coronavirus negative A72 cells; H₂O: PCR mix without DNA; T5 – T21: Tracheal samples of study dogs. The agarose gel electrophoresis shows PCR products of the expected size of 442bp for the positive control (BCV) and samples T12 and T21.

Figure 12

Comparison of the prevalence of respiratory disease in two groups of dogs.

Dogs in group 1 were positive for serum antibodies to respiratory coronavirus on day of entry into the kennel, dogs in group 2 were negative.

5 The graph shows the percentage of dogs developing respiratory disease in group 1 compared to group2 ($p<0.001$). n is the total number of dogs in each group.

Figure 13

Partial nucleotide sequence (497 residues) of the CRCV

10 hemagglutinin/esterase (HE) gene (SEQ ID NO: 21). The sequence corresponds to nucleotides 418 to 914 of the HE genes of BCV (GenBank M84486) and HCV OC43 (GenBank Accession No. M76373).

Figure 14

Partial amino acid sequence (165 residues) of the CRCV HE protein (SEQ

15 ID NO: 22), derived from the nucleotide sequence of Figure 13. This sequence corresponds to amino acid residues 140 to 304 of BCV (GenBank M84486) and HCV OC43(GenBank Accession No. M76373).

Figure 15

CLUSTAL X (1.8) multiple sequence alignment of a 497 nucleotide partial

20 sequence of the hemagglutinin/esterase (HE) gene of CRCV (canine respiratory coronavirus, SEQ ID NO: 21) with BCV (bovine coronavirus strain LY138, (SEQ ID NO: 23, taken from Genbank Accession No. AF058942), OC43 (human coronavirus strain OC43, SEQ ID NO: 24 taken from Genbank Accession No. M76373), HECV (human enteric coronavirus, SEQ ID NO: 25, taken from Genbank Accession No. L07747), and HEV

(hemagglutinating encephalomyelitis virus, SEQ ID NO: 26, taken from Genbank Accession No. AF481863).

Figure 16

CLUSTAL X (1.8) multiple sequence alignment of a 165 amino acid partial sequence of the HE protein of CRCV (canine respiratory coronavirus, (SEQ ID NO: 22) with BCV (bovine coronavirus strain LY138, SEQ ID NO: 27, taken from Genbank Accession No. AF058942), OC43 (human coronavirus strain OC43, SEQ ID NO: 28, taken from Genbank Accession No. M76373), HECV (human enteric coronavirus, SEQ ID NO: 29, taken from Genbank Accession No. L07747), and HEV (hemagglutinating encephalomyelitis virus, SEQ ID NO: 30, taken from Genbank Accession No. AF481863). The three CRCV-specific amino acids F, N and L are indicated in bold and are underlined.

Figure 17

RT-PCR using consensus primers HE1 (SEQ ID NO: 38) and HE2 (SEQ ID NO: 39) directed to the HE gene of BCV and HCV (strain OC43). The agarose gel electrophoresis shows a PCR product of the expected size of 497bp for the BCV positive control and for four tracheal samples from study dogs (T90, T91, T101 and T105), and not for coronavirus-negative A72 cells or the PCR mix without DNA (H_2O). 1 kb indicates a molecular size standard (Promega).

Figure 18

CRCV Spike gene cloning strategy.

Example 1: Detection of a novel coronavirus associated with canine infectious respiratory disease**Summary**

An investigation into the causes of canine infectious respiratory disease (CIRD) was carried out in a large re-homing kennel.⁵ Tissue samples taken from the respiratory tract of diseased dogs were tested for the presence of coronaviruses using RT-PCR with conserved primers for the polymerase gene. Sequence analysis of four positive samples showed the presence of a novel coronavirus with high similarity to both bovine and human coronavirus (strain OC43) in their polymerase and spike genes whereas there was a low similarity to comparable genes in the enteric canine coronavirus.¹⁰ This canine respiratory coronavirus (CRCV) was detected by RT-PCR in 32/119 tracheal and 20/119 lung samples with the highest prevalence being detected in dogs with mild clinical symptoms. Serological analysis showed that the presence of antibodies against CRCV on the day of entry into the kennel decreased the risk of developing respiratory disease.¹⁵

Materials and Methods**Study population**

Dogs from a well-established re-homing kennel with a history of endemic respiratory disease were monitored for this study.²⁰ On entry into the kennel, all dogs were vaccinated with KAVAK DA₂ PiP69 (Fort Dodge) a live attenuated vaccine for distemper virus, canine adenovirus type 2, canine parainfluenzavirus and canine parvovirus. Also, a killed leptospirosis vaccine was used (Fort Dodge). The health status of each dog was assessed twice a day by a veterinary clinician and the respiratory symptoms were graded as follows: 1: no respiratory signs, 2: mild cough, 3: cough and nasal²⁵

discharge, 4: cough, nasal discharge and inappetence, 5: bronchopneumonia. The overall health status of the dogs was graded as follows: 1: good health, 2: poor health, 3: very poor health. The age, breed and sex of the dogs were recorded.

5 For 119 dogs a full post mortem examination was performed. The tissue samples were stored at -70°C until further use.

Serum samples were collected from 111 dogs on day of entry into the re-homing kennel. For 81 dogs a follow-up serum was available on day 7 and for 111 dogs a serum was available on day 21 after entry.

10 Of the 111 dogs, 30 remained healthy during the 21 days between the first and the last serum sample whereas 81 dogs developed respiratory disease.

Sera from 35 dogs housed elsewhere were obtained from the diagnostic service of the Royal Veterinary College. These sera had been submitted for biochemical analysis for various reasons. Five of these sera were from 18-month-old beagles with no history of respiratory disease. Sera were 15 routinely stored at -20°C.

RNA extraction and RT-PCR

RNA was extracted from tracheal and lung tissue of 119 dogs using TriReagent (Sigma). Approximately 25-50 mg of homogenised tissue was 20 used and RNA was extracted as recommended by the manufacturer.

Synthesis of cDNA was performed using Random Hexamers (Roche) and ImPromII reverse transcriptase (Promega).

The polymerase gene of coronaviruses is known to be highly conserved, and has previously been used for phylogenetic analysis of this virus family

(Stephensen *et al.*, 1999). For the detection of coronaviruses a modification of the primers 2Bp and 4Bm directed against the polymerase gene as described by Stephensen *et al.* (1999) were used (Conscoro5: 5' -ACT-CAR-ATG-AAT-TTG-AAA-TAT-GC (SEQ ID NO: 31); and Conscoro6: 5' -TCA-CAC-TTA-GGA-TAR-TCC-CA (SEQ ID NO: 32)).

PCR was performed using Taq polymerase (Promega) in the provided reaction buffer containing a final concentration of 2.5 mM MgCl₂ and 0.5 μM of primers. For PCR with the primers Conscoro5 and Conscoro6 the following temperature profile was used: After denaturation at 95°C for 5 min, 10 cycles were carried out at 95°C for 1 min, annealing at 37°C for 1 min and extension at 72°C for 1min. This was followed by 10 cycles using an annealing temperature of 45°C, 10 cycles at an annealing temperature of 50°C and 10 cycles at an annealing temperature of 53°C followed by a final extension at 72°C for 10 min.

A 20μl fraction of the PCR product was analysed on a 1.5% agarose gel and blotted onto a nylon membrane (Roche) after electrophoresis. The nylon membrane was hybridised with an oligonucleotide probe specific for the PCR product at 37°C overnight (Probe Conscoro: AAG-TTT-TAT-GGY-GGY-TGG-GA (SEQ ID NO: 33)). The probe was 3'A-tailed with Digoxigenin-dUTP and was detected using anti-Digoxigenin conjugate and CSPD chemoluminescent substrate (Roche).

Primer sequences specific for the spike gene were derived from an alignment of the spike region of bovine coronavirus strain LY-138 (AF058942) and human coronavirus strain OC43 (L14643).

A PCR was performed with the primers Spike 1 and Spike 2, followed by a nested PCR using the primers Spike 3 and Spike 4 and 2μl of the product of the first amplification.

The numbers in brackets refer to the nucleotide position in the bovine coronavirus genome.

Spike 1: 5'-CTT-ATA-AGT-GCC-CCC-AAA-CTA-AAT (25291-25314)

Spike 2: 5' -CCT-ACT-GTG-AGA-TCA-CAT-GTT-TG (25912-25890)

5 Spike 3: 5' -GTT-GGC-ATA-GGT-GAG-CAC-CTG (25320-25339)

Spike 4: 5' -GCA-ATG-CTG-GTT-CGG-AAG-AG (25762-25742)

Oligonucleotide Spike 1 has SEQ ID NO: 34, Spike 2 has SEQ ID NO: 35, Spike 3 has SEQ ID NO: 36, Spike 4 has SEQ ID NO: 37.

10 The temperature profile used was denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 40 sec and elongation at 72°C for 1 min. The final extension was performed at 72°C for 10 min. The nested PCR produced a 442bp fragment.

15 PCR products were cloned into the pGEM-T-easy vector (Promega) and sequenced using the Thermo sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia) using Cy5 labelled primers.

Phylogenetic analysis

An alignment of the 250 bp cDNA sequence from the polymerase gene to the corresponding sequences of 11 coronaviruses was performed using 20 ClustalX (Thompson *et al.*, 1997).

The phylogenetic relationship to known coronaviruses was analysed using the Phylipl 3.6 package (Felsenstein, 1989). The alignments were followed by a bootstrap analysis using the Seqboot programme. The obtained data sets were used for a maximum parsimony analysis using the DNAPars

programme and a consensus tree was calculated using Consense. The resulting trees were drawn using the Treeview programme (Page, 1996).

ELISA

ELISA antigen for bovine coronavirus or enteric canine coronavirus (CECV) (the antigens are a preparation from virus infected cell cultures obtained from Churchill Applied Biosciences, Huntingdon, UK) was resuspended in PBS at the concentration recommended by the manufacturer and incubated on 96 well plates (Falcon) overnight at 37°C.

The plates were washed with PBS and blocked with PBS containing 5% skimmed milk powder for 30 min. The sera were diluted 1:100 in blocking buffer and incubated on the plates for 1h. After washing with PBS/ 0.05% Tween 20 (Sigma), a peroxidase labelled rabbit anti-dog IgG conjugate (Sigma) was added (1:5000 in PBS/0.05% Tween 20) for 1 h. The plates were incubated with colour substrate (OPD, Sigma) for 10 min and the reaction was stopped by adding 2M H₂SO₄. The adsorption was determined in an ELISA photometer at 492nm.

Virus culture

Virus isolation is performed on canine adult lung fibroblasts (passage 3 to 7), MDCK and A72 cells. (It is appreciated, however, that virus isolation could be performed using primary cells or cell lines such as MDCK or A72 (canine), MDBK (bovine), HRT-18 (human rectal tumour cell line) and Vero (African Green Monkey). The lung fibroblasts are maintained in MEM with 20% fetal calf serum (FCS), MDCK and A72 cells are maintained in MEM with 5% FCS. Tracheal tissue samples (approx. 25mg) are homogenised using a scalpel and mixed vigorously in 1ml MEM containing Penicillin (100U/ml), Streptomycin (0.1mg/ml), Amphotericin B

(2.5 μ g/ml) and Trypsin (1 μ g/ml). The samples are centrifuged at 13000 rpm for 10 min. and the supernatant is used to inoculate cell cultures. After 30 min. at 37°C the supernatant is removed and maintenance medium added to the cultures. The cultures are passaged three times in the absence of a cytopathic effect. Then, RNA is extracted from the cells and RT-PCR to detect the presence of CRCV is performed.

Statistical analysis

The data were analysed using the chi-square test or Fisher's exact test and p values below 0.05 were considered statistically significant.

10 Results

PCR using consensus primers for the coronavirus RNA polymerase gene

Using the primers Conscoro5 and Conscoro6, cDNA obtained from 40 tracheal samples was analysed by RT-PCR.

15 Out of these, seven were found to be positive by PCR and subsequent hybridisation (17.5%).

The PCR products were cloned and sequenced (Figures 1 and 2) and the sequence data were compared to available viral sequences using the FASTA search program (Pearson, 1990).

20 Comparison of the coronavirus cDNA polymerase sequence obtained from four of the canine tracheal samples to other coronavirus sequences revealed that they were most similar to sequence data from BCV strain Quebec and LY138 (Genbank Accession Nos. AF220295 and AF058942, respectively) and human coronavirus strain OC43 (Genbank Accession No. AF124989).

The similarity in the analysed 250 bp sequence was 98.8% for BCV Quebec, and 98.4% for BCV LY138 and the HCV pol genes, whereas it was only 68.53% for CCV strain 1-71 pol gene (Figures 6 and 7).

An alignment of the novel sequence with the corresponding sequences of 11 5 coronaviruses and phylogenetic analysis using the maximum parsimony method resulted in the consensus tree shown in Figure 5. The cDNA sequence obtained from a tracheal sample (T101) was found on a common branch with bovine coronavirus, human coronavirus-OC43 and hemagglutinating encephalomyelitis virus.

10 The virus was called canine respiratory coronavirus (CRCV).

PCR using primers for the spike gene

For further analysis of the RNA sequence of CRCV, an alignment of the RNA for the spike gene of the bovine coronavirus LY 138 strain (AF058942) and the human coronavirus OC43 strain (L14643) was 15 performed using Clustal X (Thompson *et al.*, 1997). Consensus regions were chosen for the selection of the nested primer sets Spike 1-2 and Spike 3-4 (Figure 11). PCR analysis was performed with the cDNA obtained from 119 tracheal and lung samples using these nested primers.

In total 32 tracheal samples (26.9%) and 20 lung samples (16.8%) were 20 found positive by nested PCR. For eight dogs a positive PCR result was obtained for both, trachea and lung.

Sequence analysis of the PCR products obtained from tissues of six different dogs showed identical DNA sequences for these cDNAs (Figures 3 and 4). A comparison to known coronavirus spike sequences using the 25 FASTA program revealed a 98.1% similarity to bovine coronavirus and a 97.8% similarity to human coronavirus OC43 (Figures 9 and 10).

PCR using primers for the HE gene

Bovine coronavirus and other gorup II coronaviruses contain an additional structural protein, the hemagglutinin/esterase (HE). Because of the high similarity of CRCV with BCV, we analysed the presence of an HE gene in
5 CRCV.

An alignment of the HE genes sequences of BCV and HCv OC43 was used to design the primers HE1 and HE2 (Table 2). Four tracheal samples that had previously been identified as positive for coronavirus RNA by RT-PCR with primers for the S gene were tested by RT-PCR with the primer set for
10 the HE gene. All four samples showed a PCR band of the expected size after agarose gel electrophoresis (Figure 17).

Table 2: Primers designed from an alignment of the hemagglutinin/esterase genes of BCV (GenBank Accession No. M84486) and HCV OC43 (GenBank Accession No. M76373)

15

Name	Sequence	Location in BCV HE gene
HE 1	5'-TAT-CGC-AGC-CTT-ACT-TTT-GT	418-437
HE 2	5'-ACC-GCC-GTC-ATG-TTA-TCA-G	914-896

Primer HE1 has SEQ ID No: 38 and HE2 has SEQ ID No: 39. The sequence of the CRCV PCR product obtained using primers HE 1 and HE 2 is given in Figure 13 (SEQ ID No: 21), and its predicted amino acid
20 sequence is listed in Figure 14 (SEQ ID No: 22). A comparison of these nucleotide and amino acid sequences with the corresponding fragments of other related coronaviruses is shown in Figures 15 and 16. Three amino acids were shown to be unique to CRCV, as shown in Table 3.

Table 3: Unique amino acids in CRCV HE gene

Amino acid in CRCV	Amino acid in BCV/HECV/ HCV/ HEV	Position in BCV/HECV/ HCV/ HEV	Position in PCR product HE1-HE2
F (Phe)	L (Leu)	235	96
N (Asn)	T (Thr)	242	103
L (Leu)	V (Val)	253	114

The amino acid positions in BCV, HECV, HCV and HEV are numbered from the initial M (which is number 1) at the start of the BCV and HCV OC43 HE proteins (GenBank Accession Nos. M84486 and M76373, respectively).

5 Association of PCR positive samples with respiratory signs

Using primers for the spike gene, tracheal and lung samples from 119 dogs were analysed by RT-PCR for CRCV. Of these 42 were from dogs with no respiratory signs (grade 1), 18 dogs had shown mild respiratory signs (grade 2), 46 had shown moderate (grade 3) and 13 severe respiratory signs (grades 10 4 and 5). Grades 4 and 5 were merged due to the low case numbers in these groups.

Table 4 shows the PCR results for coronavirus in dogs with different grades of respiratory disease. Specifically, Table 4 shows the RT-PCR results from tracheal and lung samples of 119 dogs with different respiratory signs (none to severe) using a nested PCR directed against the coronavirus spike gene as well as the number of positive samples out of total sample number and the percentage of positive samples (in brackets).

Table 4: RT-PCR results for tracheal and lung samples

Respiratory signs	Trachea: Positive samples	Lung Positive samples	Trachea and lung Positive samples
None	11/42 (26.2%)	8/42 (19.1%)	2/42
Mild	10/18 (55.6%)	4/18 (22.2%)	4/18
Moderate	9/46 (19.6%)	8/46 (17.4%)	2/46
Severe	2/13 (15.4%)	0/13	0/13

Establishment of a serological assay for CRCV

Because of the homology of the spike cDNA of CRCV to the spike region of bovine coronavirus, an ELISA antigen for BCV was used for serological analysis of CRCV.

Sera from five dogs with no history of infectious respiratory disease that had not been housed in the investigated kennel were tested. The OD values ranged from -0.013 to 0.39 with an average OD value of 0.154. Furthermore, sera from 30 dogs admitted to a veterinary clinic for various reasons were tested for antibodies to coronavirus. Of these, 20 samples showed an OD of <0.4 (-0.46 to 0.396) and 10 samples showed an OD of >1.0 (1.012 to 1.949). Samples with an OD of 0.6 or above were subsequently considered positive.

Comparison of the immune response to CRCV of dogs with and without respiratory disease

The BCV-antigen ELISA was performed using paired sera of 111 dogs from the study kennel. Of these, 81 dogs had shown symptoms of respiratory disease during a period of 21 days and 30 had remained healthy.

Of the group of dogs with respiratory disease, 17 were positive for antibodies to CRCV on the day of entry into the kennel and 64 were negative.

Of the 64 dogs with no detectable antibodies to BCV on day one, 63 tested positive on day 21. All 46 dogs out of these 63 for which a sample on day 7 was available tested negative on day 7. Therefore 63 dogs showed a seroconversion during the study-period whereas only one dog remained negative.

Of the 31 dogs that had remained healthy, 17 had antibodies to CRCV on the day of entry. All of the 13 dogs that were negative on day 1 tested negative on day 7 but showed a seroconversion by day 21.

Thus, of 34 dogs that were positive for antibodies to CRCV on arrival in the kennel, 17 developed respiratory disease (50%) whereas of 77 dogs that were negative on arrival, 64 developed respiratory signs during the study-period (83.1%), (Figure 12).

Therefore dogs that had no antibodies to CRCV on entry into the kennel had an increased probability of developing respiratory disease ($p<0.001$).

Only one out of the 77 dogs that were negative on arrival remained negative during the study period of 21 days whereas 76 dogs showed a seroconversion.

Serology using canine enteric coronavirus (CECV) antigen

An ELISA assay using a canine coronavirus antigen was performed to investigate whether CRCV showed a serological cross reaction to canine enteric coronavirus. Sera from 27 dogs, previously tested for antibodies to 5 CRCV using the BCV antigen were selected.

It was found that eight dogs had antibodies to CECV on the day of entry into the kennel, of these four also had antibodies to CRCV. Nineteen dogs were found to be negative for CECV on day 1, 17 of these were also negative for CRCV. Of the 19 negative dogs, five showed a seroconversion 10 to CECV during the 21-day period of the investigation and 17 showed a seroconversion to CRCV.

Analysis of the prevalence of respiratory disease in this group showed that six out of the eight dogs (75%) that were positive for antibodies to CECV on day 1 developed respiratory disease. Out of the group of 19 dogs that had 15 no detectable antibodies to CECV on day 1, 15 showed signs of respiratory disease (78.9%), ($p=0.594$).

Virus isolation

Tracheal tissue samples from dogs that are identified as positive for CRCV RNA by RT-PCR are inoculated on cell cultures of canine adult lung 20 fibroblasts and MDCK cells. For some samples, virus isolation is also performed on A72 cells. The cultures show no signs of a cytopathic effect during three passages. After several passage, RNA is extracted from the cultures and tested for the presence of CRCV RNA by RT-PCR.

Discussion

This study reports the detection of a novel coronavirus, CRCV, in kennelled dogs with respiratory disease.

Coronaviruses have been reported to cause respiratory disease of man,
5 cattle, swine and poultry, but their presence in the respiratory tract of dogs
and a possible association with canine infectious respiratory disease (CIRD)
has not been determined.

Dogs were investigated from a kennel in which CIRD was endemic and
could not be controlled by the use of vaccines recommended against CIRD.

10 Samples taken from the respiratory tract of these dogs were examined using
RT-PCR primers directed to the conserved polymerase gene of
coronaviruses (Stephensen *et al.*, 1999).

Initially, seven tracheal samples were found to be positive; the sequence of
the RT-PCR products was determined and compared to all available
15 coronavirus polymerase gene sequences. This analysis revealed that the
cDNA sequence obtained from the canine samples had the highest similarity
to the polymerase gene of bovine coronavirus (98.8%) and human
coronavirus OC43 (98.4%) but only a very low similarity to the polymerase
gene of the enteric canine coronavirus (strain 1-71, 68.53% similarity).

20 A phylogenetic analysis was performed using the polymerase sequences of
eleven additional coronaviruses. The coronavirus detected in the respiratory
tract of dogs (CRCV) was located on a common branch with three group 2
viruses: BCV, HCV strain OC43 and HEV. However, canine enteric
coronavirus, a group 1 coronavirus, was shown to be only distantly related.

Canine respiratory coronavirus therefore is a novel coronavirus of dogs that is most closely related to BCV and HCV-OC43, both of which are known to cause respiratory disease.

To obtain more sequence information and to further determine the 5 relationship to other coronaviruses using a more variable gene, a part of the spike gene was analysed. Since CRCV had been shown to be most similar to BCV and HCV-OC43, an alignment of the sequences of their spike genes was used to design a nested set of primers. Nested primers were chosen to achieve a more sensitive assay.

10 Sequencing of the products of this RT-PCR confirmed the high similarity of CRCV with BCV and HCV-OC43.

The presence of antibodies to CRCV was analysed using an ELISA based on a BCV antigen because of the high sequence similarity of the two viruses in the spike cDNA. The ELISA results confirmed the presence of a virus 15 similar to BCV in the study population.

The prevalence of antibodies was 30% at the time of entry into the kennel and 99% after 21 days.

Interestingly and unexpectedly, serological analysis revealed that dogs with 20 antibodies to CRCV on day of entry into the kennel developed respiratory disease less frequently than dogs without antibodies ($p<0.001$). Therefore the presence of antibodies to CRCV had a protective effect against respiratory disease in this population.

Almost all dogs negative on day of entry into the kennel showed a seroconversion to CRCV within three weeks, indicating that the virus is 25 highly contagious. Serology using an antigen for canine enteric coronavirus (CECV) showed a much lower prevalence of antibodies to CECV on day

21. Therefore the BCV-ELISA results did not reflect an infection with canine enteric coronavirus and the cross-reactivity between the two antigens seems to be low.

5 Serum antibodies to CRCV were present in about 30% of dogs of various origins including dogs entering a re-homing kennel as well as pet dogs. The presence of CRCV is therefore not limited to the investigated kennel and the virus seems to be established in the dog population.

By PCR, CRCV was detected in tracheal tissue and lung tissue and therefore appears to infect the upper and lower respiratory tract of dogs.
10 Within the kennelled population, CRCV-RNA was detected in 27.3% of dogs with all grades of respiratory disease as well as in 26.2% of dogs that were apparently healthy at the time of euthanasia.

CRCV-RNA was most frequently found in the trachea of dogs with mild cough (55%). Studies using the human coronavirus strain 229E have shown,
15 that coronaviruses can cause disruption of the respiratory epithelium and ciliary dyskinesia (Chilvers *et al.*, 2001). Without being bound by theory, we believe that an infection with CRCV has a similar effect, and that the virus plays an important role in the early stages of the pathogenesis of CIRD. By damaging the respiratory epithelium and disrupting ciliary clearance CRCV facilitates the entry of other viral or bacterial pathogens.
20 Therefore while CRCV infection on its own may cause only mild respiratory symptoms, in conjunction with other pathogenic agents it could lead to severe respiratory disease.

The pathogenesis of CIRD has not been thoroughly investigated since the
25 1970s when *Bordetella bronchiseptica*, canine adenovirus type 2 and canine parainfluenza were determined to be the main causes of the disease. However the vaccination of all dogs against CPIV, CAV-2 and distemper

virus did not help to control the disease in this kennel despite evidence that the majority of dogs responded to the vaccine within 21 days (data not shown).

This study shows an association of a novel canine respiratory coronavirus
5 with CIRD. The aetiology of CIRD therefore needs to be re-evaluated and the role of novel microorganisms or microorganisms previously not associated with the disease has to be established.

References

Appel, M., and Binn L.N. (1987) Canine infectious tracheobronchitis, Short review: kennel cough. In "Virus infections of carnivores" (M. Appel Ed.),
10 1st Edition, pp201-211 Elsevier Science Publishers, Amsterdam).

Bemis, D.A., Carmichael, L.E., and Appel, M.J. (1977). Naturally occurring respiratory disease in a kennel caused by *Bordetella bronchiseptica*. Cornell Vet. **67**, 282-93.

Binn, L.N., Alford, J. P., Marchwicki, R. H., Keefe, T.J., Beattie, R. J., and Wall, H.G. (1979). Studies of respiratory disease in random-source
15 laboratory dogs: viral infections in unconditioned dogs. Lab Anim Sci. **29**, 48-52

Binn, L. N., Eddy, G. A., Lazar, E. C., Helms, J., and Murnane, T. (1967).
20 Viruses recovered from laboratory dogs with respiratory disease. Proc Soc Exp Biol Med **126**, 140-5

Chilvers, M. A., McKean, M., Rutman, A., Myint, B. S., Silverman, M., and O'Callaghan, C. (2001). The effects of coronavirus on human nasal ciliated respiratory epithelium. Eur Respir J. **18**, 965-70.

Ditchfield, J., Macpherson, L. W., and Zbitnew, A. (1962). Association of a canine adenovirus (Toronto A 26/61) with an outbreak of laryngotracheitis ("kennel cough"). *Can. Vet. Jour.* **3**, 238-247

5 Felsenstein, J. (1989). PHYLIP-Phylogeny Inference Package (Version 3.2c). *Cladistics* **5**, 164-166

Ignjatovic, J., and Sapats, S. (2000). Avian infectious bronchitis virus. *Rev Sci Tech.* **19**, 493-508.

10 Karpas, A., King, N.W., Garcia, F.G., Calvo, F., and Cross, R.E. (1968). Canine tracheobronchitis: Isolation and characterization of the agent with experimental reproduction of the disease. *Proc Soc Exp Biol Med.* **127**, 45-52.

Keil, D.J., and Fenwick, B. (1998). Role of *Bordetella bronchiseptica* in infectious tracheobronchitis in dogs. *J Am Vet Med Assoc.* **15**, 200-7.

15 Lou, T.Y., and Wenner, H.A. (1963). Natural and experimental infection of dogs with reovirus, type1: pathogenicity of the strain for other animals. *Am.J.Hyg.* **77**, 293-304.

Makela, M.J., Puhakka ,T., Ruuskanen, O., Leinonen, M., Saikku, P., Kimpimaki, M., Blomqvist, S., Hyypia, T., Arstila, P. (1998). Viruses and bacteria in the etiology of the common cold. *J Clin Microbiol.* **36**, 539-42.

20 Page, R.D.M. Treeview: An application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences* 1996 **12**: 357-358

Pearson WR. Rapid and sensitive sequence comparison with FASTP and FASTA. *Methods Enzymol.* 1990;183:63-98.

Pensaert M, Callebaut P, Vergote J. Isolation of a porcine respiratory, non-enteric coronavirus related to transmissible gastroenteritis. *Vet Q.* 1986 Jul;8(3):257-61.

Randolph JF, Moise NS, Scarlett JM, Shin SJ, Blue JT, Bookbinder PR.
5 Prevalence of mycoplasmal and ureaplasmal recovery from tracheobronchial lavages and prevalence of mycoplasmal recovery from pharyngeal swab specimens in dogs with or without pulmonary disease. *Am J Vet Res.* 1993 Mar;54(3):387-91.

Spaan W, Cavanagh D, Horzinek MC. Coronaviruses: structure and
10 genome expression. *J Gen Virol.* 1988 Dec;69 (Pt 12):2939-52.

Stephensen CB, Casebolt DB, Gangopadhyay NN. Phylogenetic analysis of a highly conserved region of the polymerase gene from 11 coronaviruses and development of a consensus polymerase chain reaction assay. *Virus Res.* 1999 Apr;60(2):181-9.

15 Storz J, Purdy CW, Lin X, Burrell M, Truax RE, Briggs RE, Frank GH, Loan RW Isolation of respiratory bovine coronavirus, other cytocidal viruses, and Pasteurella spp from cattle involved in two natural outbreaks of shipping fever. *J Am Vet Med Assoc.* 2000 May 15;216(10):1599-604.

Tenant BJ, Gaskell RM, Jones RC, Gaskell CJ. Studies on the
20 epizootiology of canine coronavirus. *Vet Rec.* 1993 Jan 2;132(1):7-11.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 1997 Dec 15;25(24):4876-82.

Example 2: Cloning and expression of CRCV Spike

The CRCV Spike gene was cloned using the primers listed in Table 5 and using the following cloning strategy, which is illustrated in Figure 18.

1. The spike gene was amplified in four overlapping fragments
5 (A,B,C,D).
2. The PCR product Sp5-Sp2 (B) was joined to the product Sp1-Sp8 (C) using the *Pvu*II site in the overlap.
3. This fragment was cloned into the pT7blue2 vector (Novagen) using the restriction sites *Nco*I and *Bst*XI.
- 10 4. The PCR fragment SpFXho-Sp6 (A) was joined to BC using the restriction site *Bst*XI in the overlap and the *Xho*I site that had been incorporated into the primer SpF-Xho.
5. Fragment ABC was moved into the baculovirus transfer vector pMelBacB (Invitrogen) using the restriction sites *Xho*I and *Nco*I.
- 15 6. The PCR fragment Sp7-SpR-HisTag- Eco (D) was joined to ABC using the restriction site *Nco*I in the overlap and the *Eco*RI site that had been incorporated into the primer SpR-Eco-HisTag resulting in the complete spike gene in pMelBacB (Spike MelBac). This construct contains a HisTag (6xHis) at the C terminus of the expressed protein.
- 20 7. For mammalian expression the complete gene was moved to pSecTagA (Invitrogen) using the *Bam*HI site in pMelBacB and the *Eco*RI site at the end of ABCD resulting in the plasmid SpikeSecTag.

Construction of a recombinant baculovirus

A co-transfection was performed in Sf9 cells using the Bac-N-Blue baculovirus DNA (Invitrogen) and Spike MelBac. The resulting baculovirus (AcSpCRCV 1-11) was shown to contain a full-length insert by 5 PCR using primers (Invitrogen) located upstream and downstream of the recombination site.

Expression in mammalian cells

The plasmid Spike SecTag was transfected into BHK-21 cells using Lipofectamine (Invitrogen). Expression of the Spike protein was analysed 10 using a serum sample from a dog that had been shown to be positive for antibodies to CRCV using ELISA (BCV antigen obtained from Churchill) and a positive control serum for BCV obtained from Churchill (chicken anti BCV). The transfected cells showed a positive signal in an immunofluorescence assay using the canine or the chicken serum and a 15 FITC labelled conjugate (FITC anti-dog IgG or FITC anti Chicken IgG).

Table 5: Primers designed from an alignment of the spike genes of bovine coronavirus (GenBank accession No. AF058942) and human coronavirus, OC43 (GenBank accession No. L14643)

Name	Sequence	SEQ ID NO:	Location in BCV spike gene
Sp 1	5'-CTT-ATA-AGT-GCC-CCC-AAA-CTA-AAT	40	1637-1660
Sp 2	5'-CCT-ACT-GTG-AGA-TCA-CAT-GTT-TG	41	2258-2236
Sp 3	5'-GTT-GGC-ATA-GGT-GAG-CAC-TG	42	1666-1686
Sp 4	5'-GCA-ATG-CTG-GTT-CGG-AAG-AG	43	2107-2088
Sp 5	5'-AAC-GGT-TAC-ACT-GTT-CAG-CC	44	931-950
Sp 6	5'-CAA-GTA-AAT-GAG-TCT-GCC-TG	45	1121-1102
Sp 7	5'-GGC-TGC-CAC-CTC-TGC-TAG-TC	46	2919-2938
Sp 8	5'-ATT-GTT-AAA-TGC-ATT-AGC-AAT-AAG-C	47	3069-3045
SpF	5'-TTT-TTG-ATA-CTT-TTA-ATT-TCC-TTA-CC	48	4-29
SpR	5'-GTC-GTC-ATG-TGA-WGT-TTT-RAT-TAC	49	4089-4066
SpF-XholI	5'-AGC-TCG-AGC-TTT-TTG-ATA-CTT-TTA-ATT-TCC-TTA-CC	50	
SpR His- EcoRI	5'- TTG-AAT-TCT-TAA-TGA-TGA-TGA-TGG-TCG-TCA- TGT-GAW-GTT-TTR-ATT-AC	51	

SpF-Xhol contains a Xho I site (bold). SpR-His-EcoR I contains a 6xHisTag (double-underlined), a stop codon (underlined) and an EcoR I site (bold)

CLAIMS

1. A coronavirus Spike (S) protein, or fragment thereof, having at least 75% amino acid sequence identity with the CRCV S protein whose amino acid sequence is listed in Figure 4, and comprising at least one of the canine respiratory coronavirus (CRCV)-specific amino acids listed in Table 1.
2. A coronavirus S protein that comprises the amino acid sequence listed in Figure 4, or a variant thereof with at least 97% amino acid sequence identity with the sequence listed in Figure 4.
3. A coronavirus polymerase (pol) protein, or fragment thereof, having at least 90% amino acid sequence identity with BCV pol protein and comprising the amino acid E at the position corresponding to position 4975 in the BCV genome (Accession No. SWALL: Q91A29).
4. A coronavirus pol protein that comprises the amino acid sequence listed in Figure 2.
5. A coronavirus hemagglutinin/esterase (HE) protein, or fragment thereof, having at least 90% amino acid sequence identity with BCV HE protein and comprising one or more of F at position 235, N at position 242 and L at position 253 of the BCV HE protein (Genbank Accession No. AF058942).

25

6. A coronavirus HE protein that comprises the amino acid sequence listed in Figure 14.

7. A polynucleotide that encodes the protein according to any of Claims 1 to 6, or the complement thereof.
8. A polynucleotide according to Claim 7 comprising the nucleotide sequence listed in Figure 3.
9. A polynucleotide according to Claim 7 comprising the nucleotide sequence listed in Figure 1.
10. 10. A polynucleotide according to Claim 7 comprising the nucleotide sequence listed in Figure 13.
11. 11. A vector comprising the polynucleotide of any of Claims 7 to 10.
- 15 12. 12. A host cell comprising the vector of Claim 11.
13. 13. A method of obtaining a protein encoded by the vector of Claim 11, the method comprising culturing the host cell of Claim 12, expressing the protein in the host cell, and purifying the protein.
- 20 14. 14. A protein obtainable by the method of Claim 13.
15. 15. A host cell according to Claim 12 wherein the vector is an expression vector comprising a eukaryotic promoter operatively linked to the polynucleotide, and wherein the host cell is a eukaryotic host cell.
- 25 16. 16. A method of obtaining a glycosylated S protein encoded by the polynucleotide of Claim 7 or 8, the method comprising culturing the host cell of Claim 15, expressing the protein in the host cell, and purifying the protein.

17. A glycosylated S protein obtainable by the method of Claim 16.

18. A method of making an anti-CRCV antibody comprising (i) raising
5 an immune response to an S protein according to any of Claims 1, 2, 14 or
17 in an animal and preparing an antibody from the animal or from an
immortal cell derived therefrom, or (ii) selecting an antibody from an
antibody-display library using an S protein according to any of Claims 1, 2,
14 or 17.

10

19. A method according to Claim 18 further comprising determining
whether the antibody has greater affinity for the CRCV S protein than for
the BCV S protein.

15

20. An anti-CRCV antibody obtainable by the method of Claim 18 or 19
, that has greater affinity for the CRCV S protein than for the BCV S
protein.

20

21. A method of making an anti-CRCV antibody comprising (i) raising
an immune response to an HE protein according to Claim 5 or 6 in an
animal and preparing an antibody from the animal or from an immortal cell
derived therefrom, or (ii) selecting an antibody from an antibody-display
library using an HE protein according to Claim 5 or 6.

25

22. A method according to Claim 21 further comprising determining
whether the antibody has greater affinity for the CRCV HE protein than for
the BCV HE protein.

23. An anti-CRCV antibody obtainable by the method of Claim 21 or 22, that has greater affinity for the CRCV HE protein than for the BCV HE protein.

5 24. A method of determining whether a dog has been exposed to CRCV, the method comprising:

- (a) obtaining a suitable sample from the dog; and
- (b) identifying CRCV or an anti-CRCV antibody in the sample.

10 25. A method according to Claim 24 or 25 wherein the anti-CRCV antibody is detected using a CRCV, BCV, human coronavirus (HCV) or hemagglutinating encephalomyelitis virus (HEV) antigen.

15 26. A method according to Claim 25 wherein the suitable sample is an antibody containing sample such as serum, saliva, tracheal wash and bronchiolar lavage, and wherein identifying an anti-CRCV antibody comprises identifying an antibody that selectively binds to BCV S protein (AF058942) HCV S protein (L14643), or to a coronavirus having an S protein with at least 75% amino acid identity with CRCV S protein (Figure 20 4) or a fragment thereof.

27. A method according to Claim 25 wherein identifying an anti-CRCV antibody comprises identifying an antibody that selectively binds to BCV HE protein (AF058942) HCV HE protein (M76373), or to a coronavirus having an HE protein with at least 90% amino acid identity with CRCV HE protein (Figure 14) or a fragment thereof.

30 28. A method according to Claim 24 wherein the suitable sample is an antibody containing sample, and wherein identifying an anti-CRCV antibody comprises identifying an antibody that selectively binds to an S

protein according to any of Claims 1, 2, 11 or 14 or to an HE protein according to Claim 5 or 6.

29. A method according to Claim 24 wherein the suitable sample is a
5 lung wash, tracheal wash, tonsilar swab or a biopsy or post-mortem sample from the respiratory tract of the dog.

30. A method according to Claim 29 wherein identifying CRCV comprises identifying a nucleic acid component of CRCV.

10 31. A method according to Claim 30 wherein identifying a nucleic acid component of CRCV comprises identifying a polynucleotide that hybridises at high stringency to the BCV genome (AF058942).

15 32. A method according to Claim 30 or 31 wherein identifying CRCV comprises identifying a polynucleotide as defined in any of Claims 7 to 10.

33. A method according to Claim 29 wherein identifying CRCV comprises identifying a protein component of CRCV.

20 34. A method according to Claim 33 wherein identifying a protein component of CRCV comprises identifying a protein according to any of Claims 1, 2, 5, 6, 11 or 14.

25 35. A method according to Claim 33 or 34 wherein identifying a protein component of CRCV comprises using an antibody reactive with CRCV.

30 36. A method according to Claim 35 wherein the antibody reactive with CRCV is an anti-BCV antibody, an anti-HCV antibody, or an anti-CRCV antibody according to Claim 20 or 23.

37. An immunosorbent assay for detecting anti-CRCV S antibodies, the assay comprising:

a solid phase coated with an S protein according to any of Claims 1,
5 2, 14 or 17, or an antigenic fragment thereof, wherein anti-CRCV S
antibodies in a sample exposed to the solid phase will bind to the protein;
and

a detectable label conjugate which will bind to the anti-CRCV
antibodies bound to the solid phase.

10

38. An immunosorbent assay for detecting anti-CRCV HE antibodies, the assay comprising:

a solid phase coated with an HE protein according to Claim 5 or 6, or
an antigenic fragment thereof, wherein anti-CRCV HE antibodies in a
15 sample exposed to the solid phase will bind to the protein; and

a detectable label conjugate which will bind to the anti-CRCV
antibodies bound to the solid phase.

39. An immunosorbent assay according to Claim 37 or 38, wherein the
20 solid phase is a microtitre well.

40. An immunosorbent assay according to Claim 37 or 38, wherein the
conjugate comprises anti-dog antibody.

25 41. An immunosorbent assay according to any of Claims 37 to 40,
wherein the conjugate comprises an enzyme.

42. An immunosorbent assay according to Claim 41, wherein the
enzyme is horseradish peroxidase.

43. An immunosorbent assay according to Claim 41 or 42, further comprising a substrate for the enzyme.

44. A solid substrate with an S protein according to any of Claims 1, 2,
5 14 or 17, or an antigenic fragment thereof, attached thereto, for capturing anti-CRCV S antibodies from a liquid sample, wherein anti-CRCV S antibodies in a sample exposed to the solid substrate will bind to the S protein.

10 45. A solid substrate with an HE protein according to Claim 5 or 6, or an antigenic fragment thereof, attached thereto, for capturing anti-CRCV HE antibodies from a liquid sample, wherein anti-CRCV HE antibodies in a sample exposed to the solid substrate will bind to the HE protein.

15 46. A solid substrate according to Claim 44 or 45, wherein the solid substrate is a microtitre well.

47. A vaccine composition for vaccinating dogs comprising a coronavirus having an S protein with at 75% least amino acid identity with BCV S protein, or a coronaviral protein having at least 75% amino acid identity with a BCV protein or an immunogenic fragment thereof, or a nucleic acid encoding said coronaviral protein or immunogenic fraction thereof.

25 48. A vaccine composition according to Claim 47 wherein the coronaviral protein is a BCV, HCV, HEV or CRCV protein, or a modification thereof.

30 49. A vaccine composition according to Claim 47 or 48 wherein the coronaviral protein is an S protein.

50. A vaccine composition according to Claim 49 comprising an S protein according to any of Claims 1, 2, 14 or 17, a BCV S protein, an HCV S protein, an HEV S protein, or an immunogenic fragment thereof.

5

51. A vaccine composition according to Claim 47 or 48 wherein the coronaviral protein is a hemagglutinin-esterase protein (HE) or an integral membrane protein (M).

10 52. A vaccine composition according to Claim 51 wherein the HE protein comprises an HE protein as defined in Claim 5 or 6, a BCV HE protein, an HCV HE protein, or an immunogenic fragment thereof.

15 53. A vaccine composition according to Claim 47 wherein the virus is selected from BCV, HCV, HEV and CRCV, or a modification thereof.

54. A vaccine composition according to any of Claims 47 to 53 and also comprising a pharmaceutically acceptable adjuvant.

20 55. A vaccine composition according to any of Claims 47 to 55 further comprising any one or more of:

(a) an agent capable of raising an immune response in a dog against canine parainfluenza virus (CPIV);

25 (b) an agent capable of raising an immune response in a dog against canine adenovirus type 2 (CAV-2);

(c) an agent capable of raising an immune response in a dog against canine herpesvirus (CHV); and

(d) an agent capable of raising an immune response in a dog against *Bordetella bronchiseptica* (*B. bronchiseptica*).

56. Use of (i) a coronavirus having an S protein with at least 75% amino acid identity with CRCV S protein, or (ii) a coronavirus having an S protein with at least 75% amino acid identity with BCV S protein, or (iii) a coronavirus having an HE protein with at least 90% amino acid identity
5 with CRCV HE protein, or (iv) a coronavirus having an HE protein with at least 90% amino acid identity with BCV HE protein, or (v) a coronavirus protein having at least 75% amino acid identity with a CRCV protein or an immunogenic fragment thereof, or (vi) a coronaviral protein having at least 75% amino acid identity with a BCV protein, or an immunogenic fragment thereof, or (vii) a nucleic acid encoding said coronaviral protein or immunogenic fraction thereof, in the preparation of a medicament for stimulating an immune response against CRCV in a dog.

57. Use of (i) a coronavirus having an S protein with at least 75% amino acid identity with CRCV S protein, or (ii) a coronavirus having an S protein with at least 75% amino acid identity with BCV S protein, or (iii) a coronavirus having an HE protein with at least 90% amino acid identity with CRCV HE protein, or (iv) a coronavirus having an HE protein with at least 90% amino acid identity with BCV HE protein, or (v) a coronavirus protein having at least 75% amino acid identity with a CRCV protein or an immunogenic fragment thereof, or (vi) a coronaviral protein having at least 75% amino acid identity with a BCV protein, or an immunogenic fragment thereof, or (vii) a nucleic acid encoding said coronaviral protein or immunogenic fraction thereof, in the preparation of a medicament for prophylaxis of respiratory disease in a dog.
20
25

58. Use according to Claim 56 or 57 wherein the coronaviral protein is a BCV, HCV, HEV or CRCV protein, or a modification thereof.

100

59. Use according to any of Claims 56 to 58 wherein the coronaviral protein is an S protein.

60. Use according to Claim 59 wherein the S protein comprises an S protein according to any of Claims 1, 2, 14 or 17, a BCV S protein, an HCV S protein, or an immunogenic fragment thereof.

61 Use according to any of Claims 56 to 58 wherein the coronaviral protein is HE or M.

10

62 Use according to Claim 61 wherein the HE protein comprises an HE protein according to Claim 5 or 6, a BCV HE protein, an HCV HE protein, or an immunogenic fragment thereof.

15 63. Use according to Claim 56 wherein the virus is selected from BCV, HCV, HEV and CRCV, or a modification thereof.

64. An S protein according to any of Claims 1, 2, 14 or 17 for use in medicine.

20

65. An HE protein according to Claim 5 or 6 for use in medicine.

25 66. A method of vaccinating a dog against CRCV, the method comprising administering to the dog a vaccine composition according to any of Claims 47 to 54.

30 67. A method for combating the spread of CRCV between dogs comprising determining whether a dog is infected with CRCV according to the method of any of Claims 24 to 36 and, if the dog is infected with CRCV, quarantining the dog.

68. A method for combating the spread of CRCV between dogs comprising determining whether a dog is infected with CRCV according to the method of any of Claims 24 to 36 and, if the dog is infected with CRCV,
5 vaccinating other dogs that have been, are, or are likely to be in contact with the dog.

69. A method for identifying a test vaccine capable of preventing canine infectious respiratory disease (CIRD) in dogs, comprising
10 (a) determining whether a dog has been exposed to CRCV according to the method of any of Claims 24 to 36,
(b) if the dog has not been exposed to CRCV, administering the test vaccine to the dog,
(c) inoculating the dog with CRCV, and
15 (d) determining whether the dog develops CIRD,
wherein the absence of CIRD in step (d) indicates that the test vaccine is capable of preventing CIRD.

70. A vaccine identified by the method of Claim 69.
20
71. The *E. coli* strain Spike D-1 CRCV, containing a plasmid whose insert contains a portion of the CRCV S cDNA, as deposited by the Royal Veterinary College at the NCIMB under Accession number NCIMB 41146 on 25 July 2002.

25
72. The plasmid contained in *E. coli* strain Spike D-1 CRCV, deposited by the Royal Veterinary College at the NCIMB under Accession number NCIMB 41146 on 25 July 2002.

73. A kit of parts for the immunosorbent assay according to any of Claims 37-43, comprising a solid phase, a CRCV or CRCV-like S protein and/or a CRCV or CRCV-like HE protein for coating the solid phase, and a detectable label conjugate.

5

74. A kit of parts according to Claim 73 wherein the solid phase comprises a microtitre plate.

10 75. A kit of parts according to Claim 73 or 74 wherein the detectable label conjugate comprises an anti-dog antibody.

76. A kit of parts according to Claim 73 or 74 wherein the detectable label conjugate comprises an enzyme.

15 77. A kit of parts according to Claim 76 further comprising a substrate for the enzyme.

20 78. A kit of parts according to any of Claims 73 to 77 further comprising a positive control sample that contains an anti-CRCV S protein antibody and/or an anti-CRCV HE protein antibody.

79. A method of passively immunising a dog against CRCV, comprising administering an antibody that reacts with CRCV to the dog.

25 80. A method according to Claim 79 wherein the antibody that reacts with CRCV comprises an anti-BCV antibody, an anti-HCV antibody, or an anti-CRCV antibody.

81. A method according to Claim 79 or 80 wherein the antibody that reacts with CRCV comprises an anti-S protein antibody or an anti-HE protein antibody.

82. A method according to any of Claims 79 to 81 wherein the antibody that reacts with CRCV comprises the anti-CRCV antibody according to Claim 20 or 23.

83. Use of an antibody that reacts with CRCV in the preparation of a medicament for passively immunising a dog against CRCV.

84. Use according to Claim 83 wherein the antibody that reacts with CRCV is an anti-BCV antibody, an anti-HCV antibody, or an anti-CRCV antibody.

85. Use according to Claim 83 or 84 wherein the antibody that reacts with CRCV is an anti-S protein antibody or an anti-HE protein antibody.

86. Use according to any of Claims 83 to 85 wherein the antibody that reacts with CRCV comprises the anti-CRCV antibody according to Claim 20 or 23.

87. A method of diagnosing CIRD the method comprising determining whether a dog has been exposed to CRCV according to the method defined in any one of Claims 24 to 36.

1/40

FIGURE 1

ctcagatgaa tttgaaatat gctatttagtg ctaagaatag agcccgcact gttgctggtg 60
tttccatact tagtactatg actggcagaa tgtttcatca aaaatgttg aaaagtatacg 120
cagctacacg tggtgttcct gttgttatag gcaccactaa attttatggc ggctgggatg 180
atatgttacg tcgcattatt aaagatgttgc acaatcctgt acttatgggt tgggattatc 240
ctaagtgtga 250

FIGURE 2

QMNLKYAISA KNRARTVAGV SILSTMGRM FHQKCLKSIA ATRGVPVVIG TTKFYGGWDD 60
MLRRLIKDVE NPVLMGWDYP KCE 84

2/40

FIGURE 3 (Page 1 of 2)

atgttttga tacttttaat ttccttacca atggctttg ctgttatagg agatttaag	60
tgtactacgg tttccatcaa tgatgttgc accggtgctc cttctattag cactgatgtt	120
gtcgatgtta ctaatggttt aggtacttat tatgttttag atcgtgtgta tttaaataact	180
acattgttgc ttaatggta ttatcctact tcaggttcta catatcgtaa tatggcactg	240
aagggactt tactattttag cacactatgg tttaaaccac cattttttc tgattttatt	300
gatggtgttt ttgctaaggta aaaaaataacc aaggttttaa aagatggtgtt agtgtatagt	360
gagtttcctg ctataactat aggtacttact tttgtaaata catcctatag tgtggtagta	420
caaccacata ctactaattt agataataaa ttacaaggta tccttagagat ctctgtttgc	480
cagtatacta tgtgcgatta cccacatacg atgtgtcatc ctaatctggg taataaacgc	540
atagaactat ggcattggta tacaggtgtt gtccctgtt tatataagcg taatttcaca	600
tatgtatgtta atgctgatta ttgttattcc catttttatac aagaagggtt tactttttat	660
gcataattttt cagacactgg tgggttact aagtttctgt ttcatgttta tttaggcacg	720
gtgctttcac attattatgt catgccctt acttgaataa gtgctatgac tttagaatac	780
tgggttacac ctctcacttt taaacaatat ttactcgctt tcaatcaaga tgggttatt	840
tttaatgctg ttgattgtta gagtgatttt atgagtgaga ttaagtgtaa aacactatct	900
atagcaccat ctactgggtt ttatgaatta aacggttaca ctgttcagcc aattgcagat	960
gtttaccgac gtatacctaa tcttcccgat tgaatatacg aggctggct taatgataag	1020
tcgggtgcctt ctccattaaa ttgggaacgt aagacctttt caaattgtaa tttaatatacg	1080
agcagcctga tgtttttat ccaggctgac tcgtttactt gtaataatatacg tgggttatt	1140
aagatatacg gtatgtgtt ttgcagcata actatagata agtttgcata acccaatgg	1200
aggaagggtt acctacaaat gggcaatttgc ggctatttgc agtctttaa ctatagaatt	1260
gatactactg ctacaagtttgc tcaatgttatac tgcgttagtaa tgggttatt	1320
agcaggttta atccttctat ttggaaatagg agatttgggt ttacagaaca atctgttttt	1380
aagcctcaac ctgttaggtt ttactgtat catgtatgttgc ttatgcaca acattgtttt	1440
aaagctccca caaatttctg tccgttaaa ttgaatgggt ctgtgtgtt aggttaggtt	1500
tttggtagat atgctggta taaaatagt ggtataggca ctgttcctgc aggtactaat	1560
tatataactt gttataatgc taaccaatgt gattgtttgt gcactccaga ccctatttt	1620
tctaaatcta cagggccta taatgtcccc caaactaaat acttagttgg cataggttag	1680
cactgttctg gtcttgctat taaaagtgtat tattgtggag gcaatcccttgc tacttgccaa	1740
ccaaaagcat ttttgggttgc gtctgtggac tcttgcatac aagggatag gtgtatatt	1800
tttgcataatt ttatgttgc tgggttaat agtggacta ctgttctac tgatttacaa	1860
aaatcaaaaca cagacataat tcttgggttgc tgggttaatt atgatcttgc tggatttaca	1920
ggccaaggta ttttgggttgc ggttaatgcg acttattata atagttggca gaacctttt	1980
tatgattcta atggtatct ctatggttttt agggactact taacaaacag aacttttatg	2040
atcgttagtt gctatagcgg tcgtgtttca gcccccttgc actctaactc ttccgaacca	2100
gcattgtat ttccggatata taaatgcatac tgcgttttataataactct ttccacgacag	2160
ctgcaaccta ttaactatattt tgatagttat ctgggttgc ttgtcaatgc tgataatagt	2220

3/40

FIGURE 3 (Page 2 of 2)

acttcttagtt ctgttcaaaccatgtgatctc acagtaggta gtggttactg gggggattac	2280
tctacacaaa gacgaagtgc tagaacgatt accactggtt atcggtttac taatttttag	2340
ccatTTactg ttaatccagt aaatgatagt ttacaccctg taggtggttt gtatgaaatt	2400
caaatacctt cagagttac tataggtaat atggaggagt ttattcaaac aagatctcct	2460
aaagttacta ttgattgtcc tgTTTTGTC tgggtgatt atgcagcatg taaatcacag	2520
ttggttgaat atggtagttt ttgtgacaat attaatgcta tactcacaga agtaaatgaa	2580
ctacttgaca ctacacagtt gcaagtagct aatagttaa tgaatggtgt cacttttagc	2640
actaagctta aagatggctt taatttcaat gtagatgaca tcaattttc ccctgttatta	2700
ggttggTTtag gaagcgaatg taataaaagtt tccagtagat ctgctataga ggattttactt	2760
ttttctaaag taaagttatc tgatgttggt ttgtttagt cttataataa ttgtactgga	2820
ggtgccgaaa ttagggacct catttggcgtg caaagttata atggtatcaa agtggcct	2880
ccactgctct cagaaaatca gatcagtggta tacactttgg ctgccacctt tgctagctg	2940
tttcctcctt ggtcagcagc agcaggcgta ccattttatt taaatgtca gtatgttatt	3000
aatggtatttgcgttaccat ggatgtgcta actcaaaatc aaaagcttat ttctaatgca	3060
tttaacaatg cccttgatgc tattcaggaa gggTTTgatg ctaccaattc tgcttagtt	3120
aaaattcaag ctgttggtaa tgcaaattgct gaagctcttataaacttatt gcaacaactc	3180
tctaataaaat ttggtgctat aagtgcTTCT ttacaagaaa ttctatctatcacttgcgt	3240
cttgaagcgc aagctcagat agacagactt atcaatgggc gtcttaccgc tcttaatgct	3300
tatgtttctc aacagcttag tgattctaca ctatggaaaat tttagtgcagc acaagctatg	3360
gagaagggtta atgaatgtgt caaaaagccaa tcatacttagga taaatttttggtaatgggt	3420
aatcatatta tatcattatgc cagaatgctt ccatatggtt tgtatTTTccactttagc	3480
tatgtcccta ctaagtatgt cactgcgaag gttatcccgttgcgt ygcagggtat	3540
agaggtatag ctcctaagag tggTTTTTgttaatgtaa ataacacttg gatgttact	3600
ggtagtggtt attactaccc tgaacctata actggaaata atgtgggtgt tatgagtacc	3660
tgtgctgtta actataactaa agcaccggat gtaatgctga acatttcaac acccaaccc	3720
cctgattttta aggaagagtt ggatcaatgg tttaaaaacc aaacattaat ggcaccagat	3780
ttgtcacttg attatataaa tgTTACATTCT ttggacctac aagatgaaat gaataggta	3840
caggaggcaaa taaaagttttaa atatcatagc tacatcaatc tcaaggacat tggtacatat	3900
gaatattatg taaaatggcc ttggatgtta tggCTTTAA ttggccttgc tggcgttagct	3960
atgcttggtt tactattctt catatgctgt tgcacaggat gtgggacttag ttgttttaag	4020
aaatgcgggtg gttgttgcgttga tgattataact ggacatcagg agttagtaat caaaaacgtca	4080
catgacgact aa	4092

FIGURE 4

MFLILLISLP MAFAVIGDLK CTTVSINDVD TGAPSISTDV VDVTNGLGTY YVLDRVYLNT	60
TLLLNGYYPT SGSTYRNMAL KGTLLSTLW FKPPFLSDFI DGFAVKVNT KVIKDGVVYS	120
EFPAITIGST FVNNTSYSVVV QPHHTNLNDK LQGLLEISVC QYTMCDYPHT MCHPNLGNKR	180
IELWHWDTCV VPCLYKRNFT YDVNADYLYS HFYQEGGTFY AYFTDTGVVT KFLFHVYLGT	240
VLSHYYVMPL TCNSAMTLEY WVTPLTFKQY LLAFNQDGVI FNAVDCKSDF MSEIKCKTLS	300
IAPSTGVYEL NGYTVQPIAD VYRRIPNLPD CNIEAWLNDK SVPSPLNWER KTFNSNCNFNM	360
SSLMSFIQAD SFTCNNIDAA KIYGMCFSSI TIDKFAIPNG RKVDLQMGNL GYLQSFNYRI	420
DTTATSCQLY YNLPASNNSI SRFNPSIWNR RFGFTEQSVF KPQPVGVFTD HDVVYAQHCF	480
KAPTNFCPCK LNGSLCVGSG FGIDAGYKNS GIGTCPAGTN YLTCYNANQC DCLCTPDPFIL	540
SKSTGPYKCP QTGYLVGIGE HCSGLAIKSD YCGGNPCTCQ PKAFLGWSVD SCLQGDRCNI	600
FANFILHGVN SGTTCSTDLO KSNTDIILGV CVNYDLYGIT GQGIFVEVNA TYYNSWQNL	660
YDSNGNLYGF RDYLTNRTFM IRSCYSGRVS AGFHSNSSEP ALLFRNIKCN YVFNNNTLSRQ	720
LQPINYFDSY LGCVVNADNS TSSSVQTCDL TVGSGYWGDY STQRSSRTI TTGYRFTNFE	780
PFTVNPVNDL LHPVGGLYEI QIPSEFTIGN MEEFIQTRSP KVTIDCPVFV CGDYAAACKSQ	840
LVEYGSFCDN INAILTEVNE LLDTTQLQVA NSLMNGVTLS TKLKDGFN FN VDDINFSPVL	900
GCLGSECNKV SSRSAIEDLL FSKVKLSDVG FVDAYNNCTG GAEIRDPLICV QSYNGIKVLP	960
PLLSENOQISG YTLAATFASL FPPWSAAAAGV FFYLNQYRI NGIGVTMDVL TQNQKLISNA	1020
FNNALDAIQE GF DATNSALV KIQAVVNANA EALNNNLLQQL SNKFGAISAS LQEILSRUDA	1080
LEAQAOQIDRL INGRITALNA YVSQQLSDST LVKFSAQQAM EKVNECVKSQ SSRINFCGNG	1140
NHIISLVQNA PYGLYFIHFS YVPTKYVTAK VSPGLCIAGD RGIAPKSGYF VN VNNTWMFT	1200
GSGYYYYPEPI TGNNVVVMST CAVNYTKAPD VMLNISTPNL PDFKEELDQW FKNQTLMAPD	1260
LSLDYINVTF LDLQDEMNRQ QEAIKVLNHS YINLKDIGTY EYYVKWPWYV WLLIGLAGVA	1320
MLVLLFFICC CTGCGTSCFK KCGGCCDDYT GHQELVIKTS HDD	1363

5/40

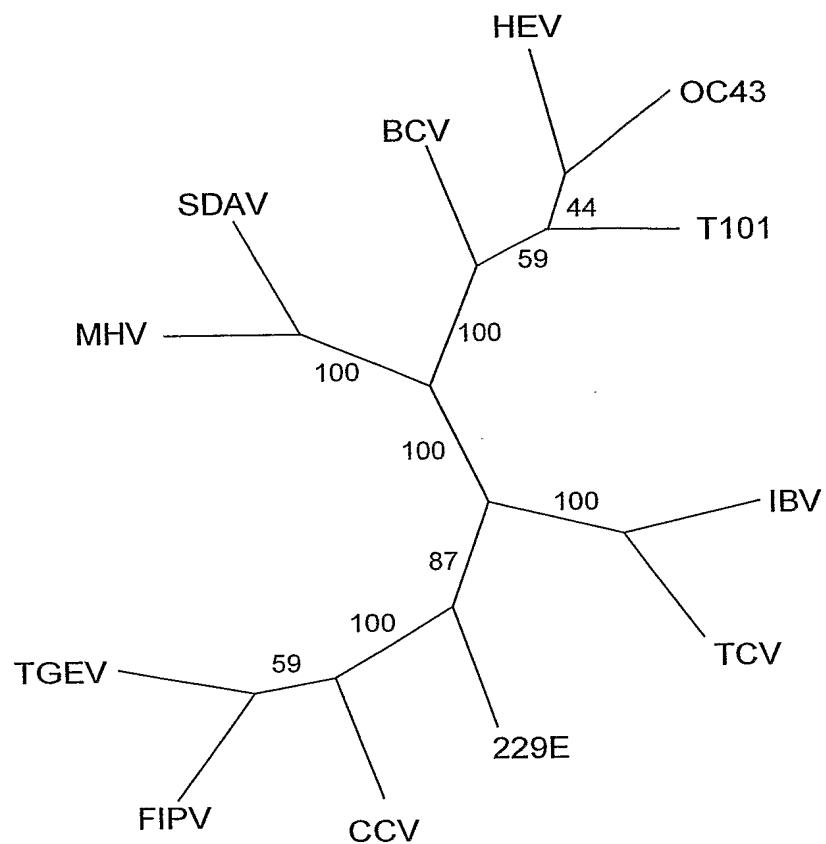
FIGURE 5

FIGURE 6

FIGURE 7

protHCVpoly	---MNLKYAISAKNRARTVAGVSILSTMTGRMFHQKCLKSIAATR
protHEVpoly	---MNLKYAISAKNRARTVAGVSILSTMTGRMFHQKCLKSIAATR
protBCVpoly	---MNLKYAISAKNRARTVAGVSILSTMTGRMFHQKCLKSIAATR
protCRCpol	--QMNLKYAISAKNRARTVAGVSILSTMTGRMFHQKCLKSIAATR
protCECpoly	MTQMNLYAISGKARARTVGVSLLSTMTRQYHQKHLKSIAATR *****.* *****.***:***** * :*** *****
protHCVpoly	GVPVVI GTTKFYGGWDDMLRRLIKD VDNPVLMGWDYPKC
protHEVpoly	GVPVVI GTTKFYGGWDDMLRRLIKD VDNPVLMGWDYPKC
protBCVpoly	GVPVVI GTTKFYGGWDDMLRRLIKD VDNPVLMGWDYPKC
protCRCpol	GVPVVI GTTKFYGGWDDMLRRLIKD VENPVLMGWDYPKC--
protCECpoly	NATVVIGSTKFYGGWDNMLKNLMDVDNGCLMGWDYPKC-- ... ***:*****:***:.*:***:*** *****

FIGURE 8 (Page 1 of 9)

FIGURE 8 (Page 2 of 9)

FIGURE 8 (Page 3 of 9)

FIGURE 8 (Page 4 of 9)

FIGURE 8 (Page 5 of 9)

FIGURE 8 (Page 6 of 9)

CRCVspike	ACAATATTAATGCTATACTCACAG-AAGT-----AAATGAACACTTGACACTA
CECVspike	AAAATATTGAGCAAGCGCTTGCAATGAGTGCCAGCCTGAAAACATGGAAGTTGATTCCA * * ***** * ** ** *** *** * ***** * *
CRCVspike	CACAGTTGCAAGTAGCTAATAGTTAATGAATGGTGTCACTCTTAGCACTAACGCTAAAG
CECVspike	TGTTGTTGTTTCAGAAAATGCCCTTA-AATTGGCATCTGTTGAGGCAGTCAATAGTACA *** ** *** * * * *** ** * ** * * *
CRCVspike	ATGGCTTTAATTCAATGTAGATGACAT---CAATT---TTCCCTGTATTAGGTGTTG
CECVspike	GAACATTTAGATCCTATTACAAAGAACATGGCCTAACATAGGTGGTTCTGGCTAGGAGGT **** * * **** * ** ** * * ** **** ***
CRCVspike	TTAGGAAGCGAAT-----GTAATAA-AGTTCCAGTA--GATCTGCTATAGAGGAT
CECVspike	CTAAAAGACATACCTCCGTCCCATAATAGCAAACGTAAGTATCGTTCTGCTATAGAACGAC ** * * * ***** * *** * ***** * ***** * ***
CRCVspike	TTACTTTCTAAAGTAAAGTTATCTGATGTTGGTTTGATGC---TTATAATAAT
CECVspike	TTGCTTTTGATAAAAGTTGTAACCTCTGGTCTAGGTACAGTTGATGAAGATTATAAACGTT ** ***** ***** *** * *** ***** ***** * *
CRCVspike	TGTACTGGAGGTGCCGAAATTAGGGACCTCATTGTGTGCAAAGTTATAATGGTATCAAA
CECVspike	TGTACAGGTGGTTATGACATAGCTGACTTAGTTGTGCACAATATTACAATGGCATCATG ***** * *** ** * *** * ***** *** *** ***** ***
CRCVspike	GTGTTGCCTC-CACTGCTCTCAGAAAATCAGATCAGTGGATACACTTGGCTGCCACCTT
CECVspike	GTTCTACCTGGTGTGCTAAT-GATGACAAGATGACTATGTACACAGCCTCTTGCAGG ** * *** *** * * *** * ***** ** *
CRCVspike	TGCTAGTCTGTTCTCC-TTGGTCAGCAGCA--GCAGGGTACCATTTATTAAATGTTG
CECVspike	TGGTATAGCATTAGGTGCACTAGGTGGTGGCGCCGTGGCTATACCTTTGCAGTAGCAGT ** * * * * * * * * *** *** ** **
CRCVspike	TCAGTATCGTATTAATGGTATTGGTGTACCATGGATGTGCTAACTCAAAATCAAAAGCT
CECVspike	TCAGGCTAGACTTAATTATGTTGCTCTACAAACTGATGTATTGAACAAAACCAGCAGAT **** * * ***** * *** * * * ***** * * *** *** ***

FIGURE 8 (Page 7 of 9)

CRCVspike	TATTTCTAATGCATTAACAATGCCCTGATGCTATT-----CAGGAAGGGTT--
CECVspike	CCTGGCTAATGCTTCAACCAAGCTATTGGTAACATTACACAGGCATTGGTAAGGTAA
	* ***** * *** * * *** * ***
	*** * ***
CRCVspike	TGATGCTA-----CCAATTCTGCT-----TTAGTTAAAAT
CECVspike	TGATGCTATACATCAAACATCACAAGGTCTGCCACTGTTGCTAAAGCATTGGCAAAAGT
	***** * *** * ***
	*** * ***
CRCVspike	TCAAGCTGTTGTTAATGCAAATGCTGAAGCTCTTAATAACTTATTGCAACAACTCTCAA
CECVspike	GCAAGATGTTGTTAACACACAAAGGGCAAGCTTAAAGCCACCTAACAGTACAACGTGCAAAA
	**** ***** *
CRCVspike	TAAATTGGTGCTATAAGTGCTTCTTACAAGAAATTCTATCTAGACTTGATGCTCTTGA
CECVspike	TAGCTTCCAAGCCATTAGTAGTTCTATTAGTGACATTATAATAGGCTTGATGAACGTGAG
	** *
CRCVspike	AGCGCAAGCTCAGATAGACAGACTTATCAATGGCGTCTTACCGCTCTTAATGCTTATGT
CECVspike	TGCTGATGCACAAGTTGATAGGCTGATTACAGGTAGACTTACAGCACTTAATGCATTG
	*** *
CRCVspike	TTCTCAACAGCTTAGTGATTCTACACTAGTAAATTTAGTGAGCACAAGCTATGGAGAA
CECVspike	ATCTCAGACTCTAACCAAGACAAGCGGAGGTTAGGGCTAGTAGACAACATTGCCAAAGACAA
	***** * * * . *
CRCVspike	GGTTAATGAATGTGTCAAAGCCAATCATCTAGGATAAATTTGTGGTAATGGTAATCA
CECVspike	GGTTAATGAATGTGTTAGGTCTCAGTCTCAGAGATTGGATTTGTGGTAATGGTACACA
	***** *
CRCVspike	TATTATATCATTAGTGCAAGATGCTCCATATGGTTGTATTTATCCACTTA-GCTATG
CECVspike	TTTGTGTTCACTTGCAAATGCAGCACCAATGGCATGGTTCTTACACAGTGCTAT-
	* *
CRCVspike	TCCCTACTAAGTATGTCACTGCGAAGGTTAGTCCCGGTCTGTGCATYGCAGGTGATAGAG
CECVspike	TACCAACAGCTTATGAAACTGTAACAGCTTGGTCAGGTATTGTGCTTCAGATGGCGATC
	* *
	*** * *

FIGURE 8 (Page 8 of 9)

FIGURE 8 (Page 9 of 9)

CRCVspike TTTTAAGAAATGCGGTGGTGTGATGATTATACTGGACA--TCAGGAGTTAGTAATC
CECVspike TTGTTGGGAAGTTGTTGTCATTCTATTGTAGTAGAAGACAATTGAAAATTACGAACC

CRCVspike	AA----AACGTCACATGACGACTAA-----
CECVspike	AATTGAAAAAGTGCATGTCCACTAAA-----
	*** *** * * * * *

FIGURE 9 (Page 1 of 12)

FIGURE 9 (Page 2 of 12)

FIGURE 9 (Page 3 of 12)

BCVspike	ATTTAGGCACGGTGCTTCACATTATTATGTCATGCCCTTGACTTGTAAATAGTGCTATGA
HCVspike	ATTTAGGCACGGTGCTTCACATTATTATGTCCTGCCCTTGACTTGTAAATAGTGCTATGA
CRCVspike	ATTTAGGCACGGTGCTTCACATTATTATGTCATGCCCTTGACTTGTAAATAGTGCTATGA
HEVspike	ATTTAGGCACGTGCTGTCACACTATTATGTTATGCCATTGACTTGTGATAGCGCTTAT *****
BCVspike	CTTTAGAATATTGGGTTACACCTCTCACTTCTAAACAATATTTACTCGCTTCAATCAAG
HCVspike	CTTTAGAATATTGGGTTACACCTCTCACTTCTAAACAATATTTACTAGCTTCAATCAAG
CRCVspike	CTTTAGAATACTGGGTTACACCTCTCACTTTAAACAATATTTACTCGCTTCAATCAAG
HEVspike	CTTTAGAATATTGGGTTACACCTCTCACTACTAGACAATTCTTAGCCTTGACCAGG *****
BCVspike	ATGGTGTATTAAATGCTGTTGATTGTAAGAGTGATTTATGAGTGAGATTAAGTGTAA
HCVspike	ATGGTGTATTAAATGCTGTTGATTGTAAGAGTGATTTATGAGTGAGATTAAGTGTAA
CRCVspike	ATGGTGTATTAAATGCTGTTGATTGTAAGAGTGATTTATGAGTGAGATTAAGTGTAA
HEVspike	ATGGTGTATTAAATGCTGTTGATTGCTAGTGAGATTTATGAGTGAGATTAAGTGTAA *****
BCVspike	AAACACTATCTATAGCACCATCTACTGGTGTATGAATTAAACGGTTACACTGTTCAGC
HCVspike	AAACACTATCTATAGCACCATCTACTGGTGTATGAATTAAACGGTTACACTGTTCAGC
CRCVspike	AAACACTATCTATAGCACCATCTACTGGTGTATGAATTAAACGGTTACACTGTTCAGC
HEVspike	AAACTCTTCAATTACACCACCTACTGGTGTATGAACTAAACGGTTACACAGTTCAAC ****
BCVspike	CAATTGCAGATGTTACCGACGTATACTTAATCTTCCCGATTGTAATATAGAGGCTTGGC
HCVspike	CAATTGCAGATGTTACCGACGTATACTTAATCTTCCCGATTGTAATATAGAGGCTTGGC
CRCVspike	CAATTGCAGATGTTACCGACGTATACTTAATCTTCCCGATTGTAATATAGAGGCTTGGC
HEVspike	CTGTTGCCACTGTGTATCGTAGAACCTGACTTACCCAATTGCGATATCGAAGCTTGGC * ****
BCVspike	TTAATGATAAGTCTGTGCCCTCTCCATTAAATTGGAACGTAAGACCTTTCAAATTGTA
HCVspike	TTAATGATAAGTCGGTGCCCTCTCCATTAAATTGGAACGTAAGACCTTTCAAATTGTA
CRCVspike	TTAATGATAAGTCGGTGCCCTCTCCATTAAATTGGAACGTAAGACCTTTCAAATTGTA
HEVspike	TTAATTCTAAGACCCTTCTCGCCTCTTAATTGGAACGTAAGACCTTTCAAATTGTA *****

FIGURE 9 (Page 4 of 12)

FIGURE 9 (Page 5 of 12)

FIGURE 9 (Page 6 of 12)

FIGURE 9 (Page 7 of 12)

FIGURE 9 (Page 8 of 12)

FIGURE 9 (Page 9 of 12)

BCVspike	AAGTGTTCGCCTCCACTACTCTCAGAAAATCAGATCAGTGGATAACACTTGGCTGCTACCT
HCVspike	AAGTGTTCGCCTCCACTGCTCTCAGAAATCAGATCAGTGGATAACACTTGGCTGCCACCT
CRCVspike	AAGTGTTCGCCTCCACTGCTCTCAGAAAATCAGATCAGTGGATAACACTTGGCTGCCACCT
HEVspike	AAGTGTTCGCCTCCATTGTTATCTGAAAATCAGATTAGTGGTTACACTCGGCAGCCACCG *****
BCVspike	CTGCTAGTCTGTTCCCTCCTGGTCAGCAGCAGCAGGCGTACCATTATTTAAATGTTC
HCVspike	CTGCTAGTCTGTTCCCTCCTGGTCAGCAGCAGCAGGCGTACCATTATTTAAATGTTC
CRCVspike	TTGCTAGTCTGTTCCCTCCTGGTCAGCAGCAGCAGGCGTACCATTATTTAAATGTTC
HEVspike	CTGCTAGCCTATTCCCTCCCTGGACAGCTGCAGCAGGCGTACCATTATTTAAATGTTC *****
BCVspike	AGTATCGTATTAATGGGATTGGTGTACCATGGATGTTAAGTCAAAAGCTTA
HCVspike	AGTATCGTATTAATGGGATTGGTGTACCATGGATGTTAAGTCAAAAGCTTA
CRCVspike	AGTATCGTATTAATGGTATTGGTGTACCATGGATGTTAAGTCAAAAGCTTA
HEVspike	AGTATCGTATAATGGCTGGCGTCACCATGGATGTTAAGGCCAAAACCAAAAGCTTA *****
BCVspike	TTGCTAATGCATTAACAATGCCCTGATGCTATTCAAGGAAGGGTTGATGCTACCAATT
HCVspike	TTGCTAATGCATTAACAATGCCCTGATGCTATTCAAGGAAGGGTTGATGCTACCAATT
CRCVspike	TTTCTAATGCATTAACAATGCCCTGATGCTATTCAAGGAAGGGTTGATGCTACCAATT
HEVspike	TTGCTAGTGCATTAACAACGCTTGATTCTATCCAGGAAGGGTTGACGCAACCAATT ***
BCVspike	CTGCTTAGTAAATTCAAGCTGTTAATGCAAATGCTGAAGCTCTAACATTAT
HCVspike	CTGCTTAGTAAATTCAAGCTGTTAATGCAAATGCTGAAGCTCTAACATTAT
CRCVspike	CTGCTTAGTAAATTCAAGCTGTTAATGCAAATGCTGAAGCTCTAACATTAT
HEVspike	CTGCTTAGTAAATTCAAGGCTGTTAATGCAAATGCTGAAGCACTAACATTAT *****
BCVspike	TGCAACAACTCTCTAACATAGATTGGTGCTATAAGTTCTTACAAGAAATTCTATCTA
HCVspike	TGCAACAACTCTCTAACATAGATTGGTGCTATAAGTTCTTACAAGAAATTCTATCTA
CRCVspike	TGCRACAACCTCTAACATAGATTGGTGCTATAAGTGCCTTTACAAGAAATTCTATCTA
HEVspike	TGCAGCAACTCTCTAACAGATTGGTGCCATAAGTGCCTTTACAAGAAATTCTATCCA ***

FIGURE 9 (Page 10 of 12)

FIGURE 9 (Page 11 of 12)

BCVspike	GGATGTTCACTGGTAGTGGTTATTACTACCCTGAACCTATAACTGGAAATAATGTTGTTG
HCVspike	GGATGTTCACTGGTAGTGGTTATTACTACCCTGAACCCATAACTGGAAATAATGTTGTTG
CRCVspike	GGATGTTCACTGGTAGTGGTTATTACTACCCTGAACCTATAACTGGAAATAATGTTGTTG
HEVspike	GGATGTTCACTGGTAGTGGCTATTACTACCCTGAACCTATAACCCAAAATAATGTTGTTG *****
BCVspike	TTATGAGTACCTGTGCTGTTAATTACACTAAAGCACCGGATGTAATGCTGAACATTCAA
HCVspike	TTATGAGTACCTGTGCTGTTAATTACACTAAAGGCCGGATGTAATGCTGAACATTCAA
CRCVspike	TTATGAGTACCTGTGCTGTTAATTACACTAAAGCACCGGATGTAATGCTGAACATTCAA
HEVspike	TGATGAGTACGTGTGCTGTTAATTACTAAAGCACCGGATCTAATGCTGAACACATCGA *****
BCVspike	CACCCAACCTCCCTGATTTAACCGAAGAGTTGGATCAATGGTTAAAAACCAAACATCAG
HCVspike	CACCCAACCTCCATGATTTAACCGAAGAGTTGGATCAATGGTTAAAAACCAAACATCAG
CRCVspike	CACCCAACCTCCCTGATTTAACCGAAGAGTTGGATCAATGGTTAAAAACCAAACATTAA
HEVspike	CACCCAACCTTCCTGATTTCAAGGAAGATTGTATCAATGGTTAAAAACCAATCTCAT *****
BCVspike	TGGCACCAAGATTTGTCACTTGATTATATAATGTTACATTCTGGACCTACAAGATGAAA
HCVspike	TGGCACCAAGATTTGTCACTTGATTATATAATGTTACATTCTGGACCTACAAGATGAAA
CRCVspike	TGGCACCAAGATTTGTCACTTGATTATATAATGTTACATTCTGGACCTACAAGATGAAA
HEVspike	TGGCACCAAGATTTGTCATTGATTATATAATGTTACGTTCTGGACCTACAAGATGAAA *****
BCVspike	TGAATAGGTTACAGGAGGCAATAAAGTTAAATCAGAGCTACATCAATCTCAAGGACA
HCVspike	TGAATAGGTTACAGGAGGCAATAAAGTTAAATCAGAGCTACATCAATCTCAAGGACA
CRCVspike	TGAATAGGTTACAGGAGGCAATAAAGTTAAATCATAGCTACATCAATCTCAAGGACA
HEVspike	TGAATAGGTTACAAGAAGCTATAAAGTTCTAAATCATAGCTACATCAATCTCAAGGACA *****
BCVspike	TTGGTACATATGAGTATTATGTAATGGCTTGGTATGTATGGCTTTAATTGGCCTTG
HCVspike	TTGGTACATATGAGTATTATGTAATGGCTTGGTATGTATGGCTTTAATTGGCCTTG
CRCVspike	TTGGTACATATGAATATTATGTAATGGCTTGGTATGTATGGCTTTAATTGGCCTTG
HEVspike	TTGGTACATATGAGTATTATGTAATGGCTTGGTATGTATGGCTTTAATTGGCCTTG *****

FIGURE 9 (Page 12 of 12)

BCVspike	CTGGTGTAGCTATGCTTGTACTATTCTCATATGCTGTTGACAGGATGTGGGACTA
HCVspike	CTGGTGTAGCTATGCTTGTACTATTCTCATATGCTGTTGACAGGATGTGGGACTA
CRCVspike	CTGGCGTAGCTATGCTTGTACTATTCTCATATGCTGTTGACAGGATGTGGGACTA
HEVspike	CTGGTGTAGTTATGCTTGTACTATTCTCATATGCTGCTGTACAGGATGTGGGACTA
	***** * ***** ***** * ***** * ***** * ***** * ***** * *****
BCVspike	GTTGTTTAAGAAATGTGGTGGTTGTTGATGATTATAAC-----
HCVspike	GTTGTTTAAGATATGTGGTGGTTGTTGATGATTATACTGGACACCAGG-----
CRCVspike	GTTGTTTAAGAAATGCGGTGGTTGTTGATGATTATACTGGACATCAGG-----
HEVspike	GTTGTTTAAGAAATGTGGCGGTTGTTGATGATTATACTGGACACCAGGAGTTGTAA
	***** * *** * * ***** * *****
BCVspike	-----
HCVspike	-----
CRCVspike	-----
HEVspike	TCAAAACTTCACATGACGATTAATT CGT

FIGURE 10 (Page 1 of 5)

BCVspikepro -----MFLILLISLPMALAVIGDLKCTTVSINDVDTGVPSVSTDVTVDVTNGLGTYYVLDRV
 HCVspikepro -----MFLILLISLPTAFAVIGDLKCTTVSINDIDTGAPSISTDIVDTNGLGTYYVLDRV
 CRCVspikepr -----MFLILLISLPMFAVIGDLKCTTVSINDVDTGAPSISTDVVDVTNGLGTYYVLDRV
 HEVspikepro -----MFFILLITLPSVFAVIGDLKCNTSSINDVDTGVPSISSEVVVDVTNGLGTFYVLDRV
 CECVspikepr MIVLVTCILLCSYHTASSTSNNDCRQVNVTQLDGNENLIRDLFQNFKEEGTVVVGG--
 : ***: . : . : * . : : * . : . : * * * .
 BCVspikepro YLNNTLLLNGYYPTSGSTYRNMALKGTLKLSTLWFKPPFLSDFINGIFAKVKNTKVIKNG
 HCVspikepro YLNNTLLLNGYYPTSGSTYRNMALKGTLLLSRLWFKPPFLSDFINGIFAKVKNTKVIKKG
 CRCVspikepr YLNNTLLLNGYYPTSGSTYRNMALKGTLKLSTLWFKPPFLSDFIDGVFAKVKNTKVIKDG
 HEVspikepro YLNNTLLLNGYYPIGATFRNVALKGTRLLSTLWFKPPFLSPFNDGIFAKVKNSRFSKHG
 CECVspikepr YYPTEVWYNCSRTATTAYEYFSNIHAFYFDMEAMENSTGNARGKPLL FHVGEPVS--v
 * * : * . : : :: . : : .. : : . : : . : : : * : .
 BCVspikepro VMYSEFPAITIGSTFVNNTSYSVVVQPHTNLDNKLQGLLEISVCQYTMCEYPHTICHPNL
 HCVspikepro VMYSEFPAITIGSTFVNNTSYSVVVQPHTNLDNKLQGLLEISVCQYTMCEYPHTICHPNL
 CRCVspikepr VVYSEFPAITIGSTFVNNTSYSVVVQPHTNLDNKLQGLLEISVCQYTMCDYPHTMCHPNL
 HEVspikepro VIYSEFPAITIGSTFVNNTSYSIVVKPHTSFINGNLQGFLQISVCQYTMCEYPQTICHPNL
 CECVspikepr IIYISYRDDVQHRPLLKHGLVCITESRNIDYN-SFTSSQWNSICTGNDRKIPFSVIPTDN
 ::*::: . .::: . : . . . * : * . . * : : .
 BCVspikepro GNRRIELWHWDTGVVSCLYKRNFYDVN-----ADYLYFHFYQEGGTFYAYFTDTGVVT
 HCVspikepro GNRRVELWHWDTGVVSCLYKRNFYDVN-----ADYLYFHFYQEGGTFYAYFTDTGVVT
 CRCVspikepr GNKRIELWHWDTGVVPCLYKRNFYDVN-----ADYLYSHFYQEGGTFYAYFTDTGVVT
 HEVspikepro GNQRIELWHHDTDVSVCLYRRNFYDVN-----ADYLYFHFYQEGGTFYAYFTDTGFVT
 CECVspikepr GTKIYGLEWNDEFVTAYISGRSYNWNNINNNWFNNVTLLYSRSSTATWQHSAAYVYQGVSN
 *.. * * *.. : *.: . * . . * : . * : . * : . * ..
 BCVspikepro KFLFNVLGTVLSHYYVMP-----LTCNSAMTLEYWVTPLTSKQYLLAFNQDGVI F
 HCVspikepro KFLFNVLGTVLSHYYVLP-----LTCNSAMTLEYWVTPLTSKQYLLAFNQDGVI F
 CRCVspikepr KFLFHVLGTVLSHYYVMP-----LTCNSAMTLEYWVTPLTFKQYLLAFNQDGVI F
 HEVspikepro KFLFKLYLGTVLSHYYVMP-----LTCDSALSLEYWVTPLTRQFLLA FDQDGVL Y
 CECVspikepr FTYYKLNNTNGLKTYELCEDYEYCTGYATNIFAPTVGGYIPDGFSFNNWFLLTNSSTFVS
 ::: . *. * : . : . * : . : . * : . : . * : . : . .

103

118

166

171

179

192

210

235

267

FIGURE 10 (Page 2 of 5)

BCVspikepro	NAVDCKSDFMSEIKCKTLSIAPSTGVYELNGYTVQPIADVYRR-IPNLPCDCNIEAWLNDK
HCVspikepro	NAVDCKSDFMSEIKCKTLSIAPSTGVYELNGYTVQPIADVYRR-IPNLPCDCNIEAWLNDK
CRCVspikepr	NAVDCKSDFMSEIKCKTLSIAPSTGVYELNGYTVQPIADVYRR-IPNLPCDCNIEAWLNDK
HEVspikepro	HAVDCASDFMSEIMCKTSSITPPGTGVYELNGYTVQPVATVYRR-IPDLPNCDEAWLNSK
CECVspikepr	GRFVTNQPLLVNCNWLPVPSFGVAAQEFCFEGAQFSQCNGVFLNNNTDVIRFNLNFTADVQ::: .. *: .. : ::*: .. *: .. :: .. :: .. :: ..
	388
BCVspikepro	SVPSPLNWERKTFSNCNFNMSSLMSFIQADSFTCN-----NIDAAKIYGMCFSSITIDK
HCVspikepro	SVPSPLNWERKTFSNCNFNMSSLMSFIQADSFTCN-----NIDAAKIYGMCFSSITIDK
CRCVspikepr	SVPSPLNWERKTFSNCNFNMSSLMSFIQADSFTCN-----NIDAAKIYGMCFSSITIDK
HEVspikepro	TVSSPLNWERKIFSNCNFNMGRMLMSFIQADSFGCN-----NIDASRLYGMCFGSSITIDK
CECVspikepr	SGMGATVFSLNTGGCILEISCYNDIVSESSFYSYGEIPFGVTDGPRCYVLYNGTALKY : : ..* ::.. ..:: .. .** .. *...: .. ::..
	407 436 440 447
BCVspikepro	FAIPNGRKVDLQLGNLGYLQSFNYRIDTTATSCOLYYNLPAN-VSVSRFPNSTWNRRFG
HCVspikepro	FAIPNGRKVDLQLGNLGYLQSFNYRIDTTATSCOLYYNLPAN-VSVSRFPNSTWNRRFG
CRCVspikepr	FAIPNGRKVDLQMGNLGYLQSFNYRIDTTATSCOLYYNLPASN-VSISRFNPSSIWNRRFG
HEVspikepro	FAIPNSRKVDLQVGKSGYLQSFNYKIDTAVSSCOLYYSLPAAN-VSVTHYNPSSWNRRYG
CECVspikepr	FGTLPPSVKEIAISKWGQFYINGYNFFSTFPIDCISFNLTGDSGAFWTIAYTSYTEALV *. .. :: .. * : .. *..: .. . : ..*..: .. : ..
	501
BCVspikepro	FTEQSVFKPQPVGVFTDHVVYAQHCFKAPTNFCPCLDGSLCVGSGSGIDAGYKNSGIG
HCVspikepro	FTEQSVFKPQPVGVFTHDVVYAQHCFKAPTNFCPCLDGSLCVGNPGIDAGYKNSGIG
CRCVspikepr	FTEQSVFKPQPVGVFTDHVVYAQHCFKAPTNFCPCLDGSLCVGSGFGIDAGYKNSGIG
HEVspikepro	FINQS-----FGSRLHDAVYSQQCFNTPNTYCPCRT--SOCIGG-----AGTG
CECVspikepr	QVENTAIKKVTCNSHINNIKCSQLTANLQNGFYPVASSEVGLVNKSVVLLPSFYSHTSV ::: .. : * : .. : * .. : ..
	525 528 540
BCVspikepro	TCPAGTNYLTC-----NAAQCNCCLCTPDPITSKSTGPYKCPQTKEYLVGIGEHCSGLAIKS
HCVspikepro	TCPAGTNYLTC-----NAAQCDCLCTPDPITSKSTGPYKCPQTKEYLVGIGEHCSGLAIKS
CRCVspikepr	TCPAGTNYLTCY-----NANQCDCLCTPDPILSKSTGPYKCPQTKEYLVGIGEHCSGLAIKS
HEVspikepro	TCPVGGTWRKCFAAVTNATKCTCWCQPDYSTYKVNAWTCPQSKVSIQPGQHCPGLGLVE
CECVspikepr	NITIDLGMKRSGYG--QPIASTLSNITLPMQDNNTDVYCIRSNQFSVYVHSTCKSSLWDN * .. : .. : .. : .. * ..

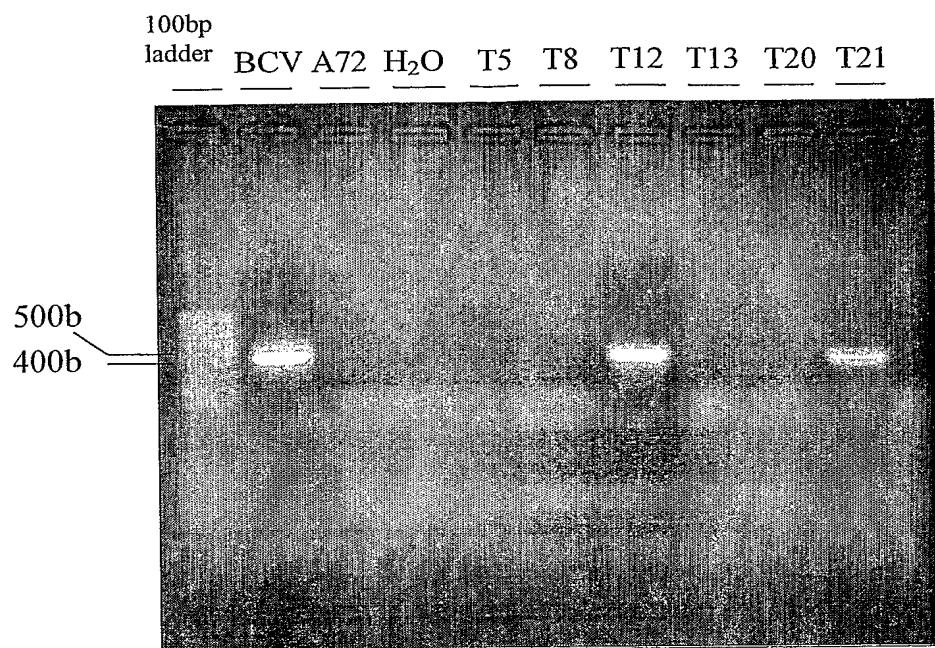
FIGURE 10 (Page 3 of 5)

FIGURE 10 (Page 4 of 5)

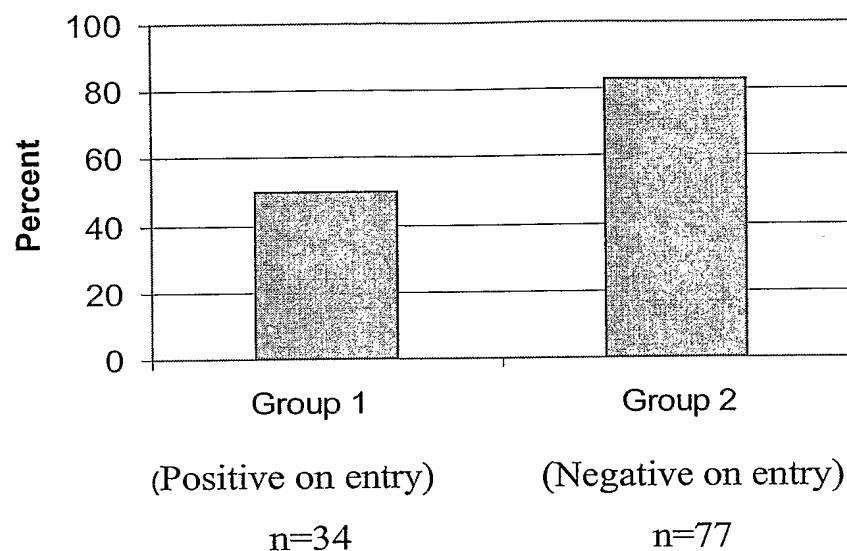
FIGURE 10 (Page 5 of 5)

BCVspikepro	ECVKSQSSRINF CGNGNHIISLVQNAPYGLYFIHFSYVPTKYVTAKVSPGLCIAGDRGIA
HCVspikepro	ECVKSQSSRINF CGNGNHIISLVQNAPYGLYFIHFSYVPTKYVTAKVSPGLCIAGDRGIA
CRCVspikepr	ECVKSQSSRINF CGNGNHIISLVQNAPYGLYFIHFSYVPTKYVTAKVSPGLCIAGDRGIA
HEVspikepro	ECVKSQSSRINF CGNGNHIISLVQNAPYGLYFIHFSYVPTKYVTAKVSPGLCIAGDIGIS
CECVspikepr	ECVRSQSQRFGFCGNGTHLFSLANAAPNGMVFHTVLLPTAYETVTAWSGICASDGDRTF ***:***.*:*****.*:***: ** *: *: :** * *.. .*: * :..
BCVspikepro	-----PKSGYFVN VNNNTWMFTGSGYYYPEPITGNNVVVMSTCAVNYTKAPDVMLNISTP
HCVspikepro	-----PKSGYFVN VNNNTWMFTGSGYYYPEPITGNNVVVMSTCAVNYTKAPDVMLNISTP
CRCVspikepr	-----PKSGYFVN VNNNTWMFTGSGYYYPEPITGNNVVVMSTCAVNYTKAPDVMLNISTP
HEVspikepro	-----PKSGYFINVNN SWMFTGSGYYYPEPITQNNVVVMSTCAVNYTKAPDMLNTSTP
CECVspikepr	GLVVKDVQLTLFRNLDDKFYLTPRTMYQPR AATSSDFVQIEGCDVL FVNATVIDLPSIIP : * *::: : * : * *.. * ..* : * * :*. : * * <i>1256</i> <i>1257</i>
BCVspikepro	NLPDFKEELDQWFKNQTS--VAPDLSLDYINVTFLDLQDEMN-----RLQE
HCVspikepro	NLHDFKEELDQWFKNQTS--VAPDLSLDYINVTFLDLQDEMN-----RLQE
CRCVspikepr	NLPDFKEELDQWFKNQTL--MAPDLSLDYINVTFLDLQDEMN-----RLQE
HEVspikepro	NLPDFKEELYQWFKNQSS--LAPDLSFDYINVTFLDLQDEMN-----RLQE
CECVspikepr	DYIDINQTVQDILENYRPNWTVP ELTIDIFNATYLNLTGEIDDLEFRSEKLHNTTVELAI : *::: : : : * . *::: * :*.*: * .*: . * *.
BCVspikepro	AIKV L N Q S Y I N L K D I G T Y E Y Y V K W P W Y V W L L I G L A G V A M L V L L F F I C C C T G C G T S C F K K C
HCVspikepro	AIKV L N Q S Y I N L K D I G T Y E Y Y V K W P W Y V W L L I G F A G V A M L V L L F F I C C C T G C G T S C F K I C
CRCVspikepr	AIKV L N H S Y I N L K D I G T Y E Y Y V K W P W Y V W L L I G L A G V A M L V L L F F I C C C T G C G T S C F K K C
HEVspikepro	AIKV L N H S Y I N L K D I G T Y E Y Y V K W P W Y V W L L I C L A G V V M L V L L F F I C C C T G C G T S C F K K C
CECVspikepr	LIDNINNTLVNLEWLNR IETYV K W P W Y V W L L I G L V V V F C I P L L L F C C C S T G C C G -CIGCL *. :*: : **: :. * ***** :. * : * : * * . *** *: GGCCDDYTGHQELVIKTSHDD----- GGCCDDYTGHQELVIKTSHDD----- GGCCDDYTGHQELVIKTSHDD----- GGCFDDYTGHQEFVIKTSHDD----- GSCCHSICSRRQFENYEPIEKVHVH----- *.* .. .::: . :.

34/40

FIGURE 11

35/40

FIGURE 12

36/40

FIGURE 13

TATCGCAGCC TTACTTTGT TAATGTACCA TATGTTATA ATGGCTCTGC ACAATCTACA 60
GCTCTTGTA AATCTGGTAG TTTAGTTCTT AATAACCCTG CATATATAGC TCGTGAAGCT 120
AATTTGGGG ATTATTATTA TAAGGTTGAA GCTGATTCT ATTTGTCAGG TTGTGACGAG 180
TATATCGTAC CACCTTGAT TTTAACGGC AAGTTTTGT CGAATACAAA GTATTATGAT 240
GATAGTCAAT ATTATTTAA TAAAGACACT GGTGTTATTT ATGGTTTCAA TTCTACTGAA 300
ACCATTAACA CTGGTTTGA TTTAATTGT CATTATTAC TTTACCCCTC TGGTAATTAT 360
TTAGCCATT CAATGAGCT ATTGTTAACT GTTCCTACGA AAGCAATCTG TCTTAATAAG 420
CGTAAGGATT TTACGCCTGT ACAGGTTGTT GACTCGCGGT GGAACAATGC CAGGCAGTCT 480
GATAACATGA CGCGGG 497

FIGURE 14

YRSLTFVNVP YVYNGSAQST ALCKSGSLVL NNPAYIAREA NFGDYYYKVE ADFYLSGCDE 60
YIVPLCIFNG KFLSNTKYDD DSQYYFNKDT GVIYGFNSTE TINTGFDFNC HYLLLPSGNY 120
LAISNELLT VPTKAICLNK RKDFTPVQVV DSRWNNARQS DNMTA 165

FIGURE 15 (Page 1 of 2)

CRCV	TATCGCAGCCTTACTTTGTTAATGTACCATATGTTATAATGGCTCTGCACAATCTACA
BCV	TATCGCAGCCTTACTTTGTTAATGTACCATATGTTATAATGGCTCTGCACAATCTACA
OC43	TATCGCAGCCTTACTTTGTTAATGTACCATATGTTATAATGGCTCTGCACAATCTACA
HECV	TATCGCAGCCTTACTTTGTTAATGTACCATATGTTACAATGGCTCTGCACAATCTACA
HEV	TATCGCAGTCTTACTTAGTTAATGTGCCATACGTTACAATGGTCAGCTCAACCCACC *****
CRCV	GCTCTTGAAATCTGGTAGTTAGTTCTTAATAACCCCTGCATATATAGCTCGTGAAGCT
BCV	GCTCTTGAAATCTGGTAGTTAGTTCTTAATAACCCCTGCATATATAGCTCGTGAAGCT
OC43	GCTCTTGAAATCTGGTAGTTAGTCCTTAATAACCCCTGCATATATAGCTCCTCAAGCT
HECV	GCTCTTGAAATCTGGTAGTTAGTCCTTAATAACCCCTGCATATATAGCTCGTGAAGCT
HEV	GCACTTTGTAAGTCTGGCAGTTAACAACTCTGCATATATAGCCCGTGAGGCT ***
CRCV	AATTTGGGGATTATTATAAGGTTGAAGCTGATTTCTATTGTCAGGTTGTGACGAG
BCV	AATTTGGGGATTATTATAAGGTTGAAGCTGATTTCTATTGTCAGGTTGTGACGAG
OC43	AACTCTGGGGATTATTATAAGGTTGAAGCTGATTTCTATTGTCAGGTTGTGACGAG
HECV	AATTTGGGGATTATTATAAGGTTGAAGCTGATTTCTATTGTCAGGTTGTGACGAG
HEV	AATGTGGGTGATTATTATAAGTCTGAAGCAGATTTCTCTCAGGTTGTGACGAG ***
CRCV	TATATCGTACCACTTGTATTAAACGGCAAGTTTGTGAAATACAAAGTATTATGAT
BCV	TATATCGTACCACTTGTATTAAACGGCAAGTTTGTGAAATACAAAGTATTATGAT
OC43	TATATCGTACCACTTGTATTAAACGGCAAGTTTGTGAAATACAAAGTATTATGAT
HECV	TATATCGTACCACTTGTATTAAACGGCAAGTTTGTGAAATACAAAGTATTATGAT
HEV	TATATCGTACCACTTGTATTAAACGGCAAGTTTGTGAAATACAAAGTATTATGAT *****
CRCV	GATAGTCATAATTAAAGACACTGGTGTATTGTTCAATTCTACTGAA
BCV	GATAGTCATAATTAAAGACACTGGTGTATTGTTCAATTCTACTGAA
OC43	GATAGTCATAATTAAAGACACTGGTGTATTGTTCAATTCTACAGAA
HECV	GATAGTCATAATTAAAGACACTGGTGTATTGTTCAATTCTACTGAA
HEV	GATAGTCATAATTAAAGACACTGGTGTATTGTTCAATTCTACTGAA *****
CRCV	ACCATTAACACTGGTTTGATTAAATTGTCATTATTTACCCCTGGTAATTAT
BCV	ACCATTACCACTGGTTTGATTAAATTGTCATTATTTACCCCTGGTAATTAT
OC43	ACCATTACCACTGGTTTGATCTTAATTGTTATTGTTACCCCTGGTAATTAT
HECV	ACCATTACCACTGGTTTGATCTTAATTGTCATTATTTAGTTCTACCCCTGGCAATTAT
HEV	ACCATTACCACTGGTTTGATCTTAATTGTCATTATTTAGTTCTACCCCTGGTAATTAT *****
CRCV	TTAGCCATTCAAATGAGCTATTGTTAATGTTACTGTTCTACGAAAGCAATCTGTCTTAATAAG
BCV	TTAGCCATTCAAATGAGCTATTGTTAATGTTACTGTTCTACGAAAGCAATCTGTCTTAATAAG
OC43	TTAGCCATTCAAATGAGCTATTGTTAATGTTACTGTTCTACGAAAGCAATCTGTCTTAATAAG
HECV	TTAGCCATTCAAATGAGCTATTGTTAATGTTACTGTTCTACGAAAGCAATCTGTCTTAATAAG
HEV	CTAGCCATTCAAATGAGCTATTGTTAATGTTACTGTTCTACGAAAGCAATCTGTCTTAATAAG *****

(Page 2 of 2)

CRCV	CGTAAGGATTTACGCCTGTACAGGTTGTTGACTCGCGGTGGAACAATGCCAGGCAGTCT
BCV	CGTAAGGATTTACGCCTGTACAGGTTGTTGACTCTCGGTGGAACAATGCCAGGCAGTCT
OC43	CGTAAGGATTTACGCCTGTACAGGTTGTTGATT CGCGGTGGAACAATGCCAGGCAGTCT
HECV	CGTAAGGATTTACGCCTGTACAGGTTGTTGACTCGCGGTGGAACAATGCCAGGCAGTCT
HEV	CGTAAGGTTTACGCCTGTACAGGTTGTTGATT CGCGGTGGAACAATGCCAGGCAATCT

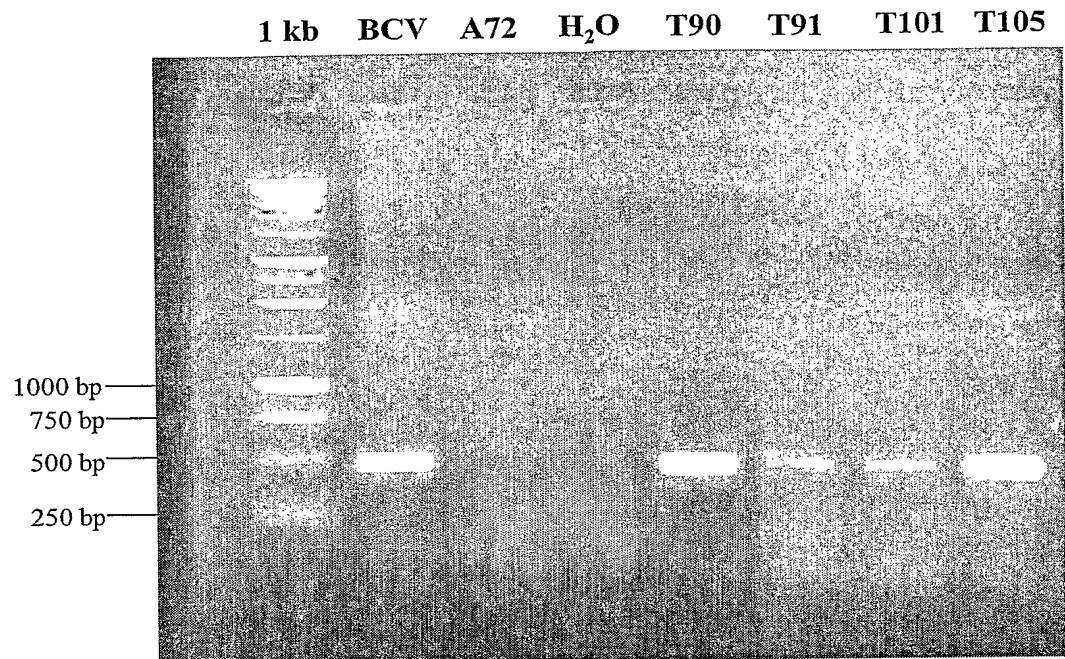
CRCV	GATAACATGACGGCGGT
BCV	GATAACATGACGGCGGT
OC43	GATAACATGACGGCGGT
HECV	GATAACATGACGGCAGT
HEV	GATAACATGACGGCAGT

FIGURE 16

CRCV	YRSLTFVNVPYVYNGSAQSTALCKSGSLVLNNPAYIAREANFGDYYYKVEADFYLSGCDE
BCV	YRSLTFVNVPYVYNGSAQSTALCKSGSLVLNNPAYIAREANFGDYYYKVEADFYLSGCDE
OC43	YRSLTFVNVPYVYNGSAQSTALCKSGSLVLNNPAYIAPQANS GDYYYKVEADFYLSGCDE
HECV	YRSLTFVNVPYVYNGSAQSTALCKSGSLVLNNPAYIAREANFGDYYYKVEADFYLSGCDE
HEV	YRSLTLVNVNPYVYNGSAQPTALCKSGSLILNNPAYIAREANVGDYYYKSEADFSLSCDE

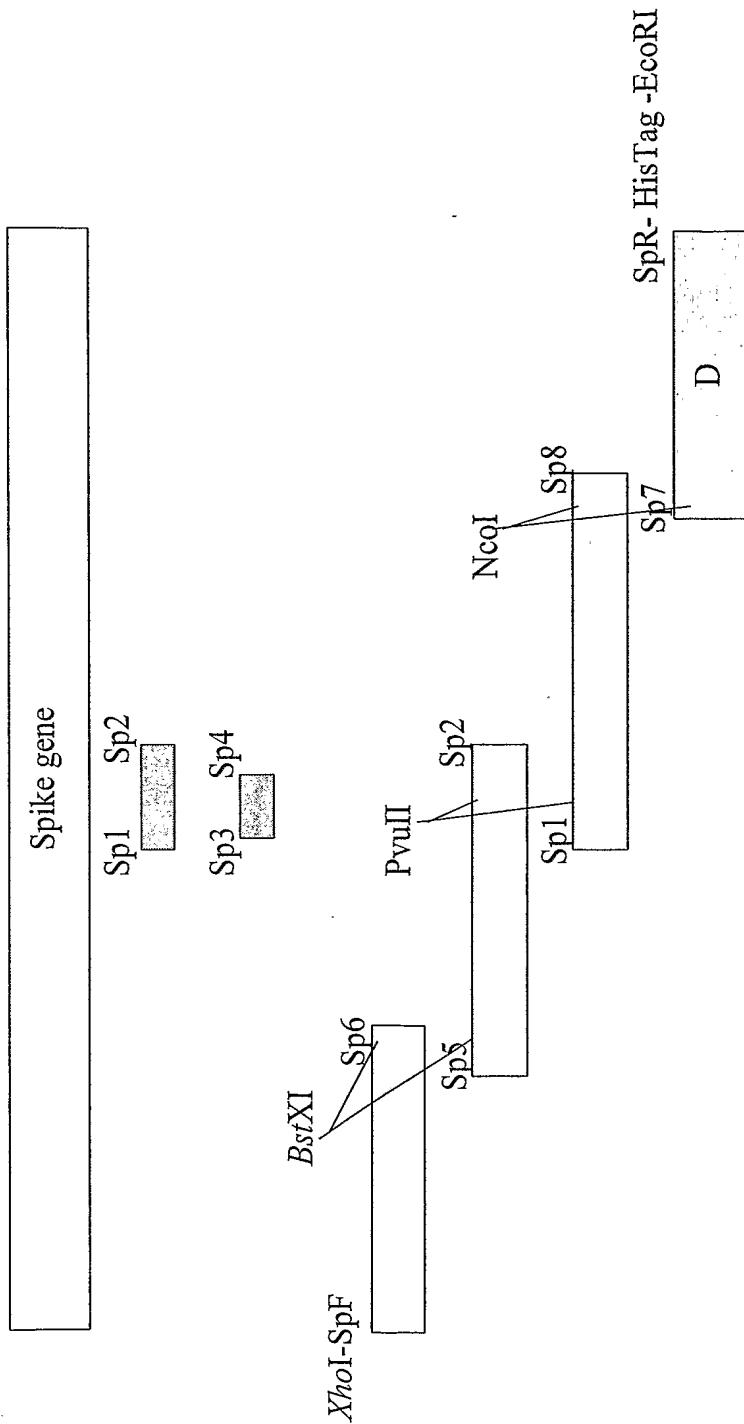
CRCV	YIVPLCIFNGKFLSNTKYYDDSQYYFNKDTGVIYGFNSTETINTGDFNCHYL L LPSGNY
BCV	YIVPLCIFNGKFLSNTKYYDDSQYYFNKDTGVIYGLNSTETITTGDFNCHYL L LPSGNY
OC43	YIVPLCIFNGKFLSNTKYYDDSQYYFNKDTGVIYGLNSTETITTGFDLNCYYL L LPSGNY
HECV	YIVPLCIFNGKFLSNTKYYDDSQYYFNKDTGVIYGLNSTETITTGDFNCHYL L LPSGNY
HEV	YIVPLCIFNGKFLSNTKYYDDSQYYFNKDTGVIYGLNSTETITTGDFNCHYL L LPSGNY

CRCV	LAISNELLTVPTKAICLNKRKDFTPVQVVDSRWNNARQSDNMTA
BCV	LAISNELLTVPTKAICLNKRKDFTPVQVVDSRWNNARQSDNMTA
OC43	LAISNELLTVPTKAICLNKRKDFTPVQVVDSRWNNARQSDNMTA
HECV	LAISNELLTVPTKAICLNKRKDFTPVQVVDSRWNNARQSDNMTA
HEV	LAISNELLTVPTKAICLNKRKVFTPQVVDSRWNNARQSDNMTA

FIGURE 17

40/40

FIGURE 18



INTERNATIONAL SEARCH REPORT

Internal	Application No
PCT/	03/02832

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/50 C07K14/165 G01N33/50 C12Q1/68 C12N7/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, Sequence Search, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>STEPHENSEN, C.B., CASEBOLT, D.B., GANGOPADHYAY, N.N.: "Phylogenetic analysis of a highly conserved region of the polymerase gene from 11 coronaviruses and development of a consensus polymerase chain reaction assay" VIRUS RESEARCH, AMSTERDAM, NL, vol. 60, no. 2, April 1999 (1999-04), pages 181-189, XP002242737 ISSN: 0168-1702 cited in the application the whole document</p> <p>-----</p> <p style="text-align: center;">-/--</p>	1-10, 24, 30, 31

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

° Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the International filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

20 October 2003

Date of mailing of the international search report

27/10/2003

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Chambonnet, F.

INTERNATIONAL SEARCH REPORT

Internal	Application No
PCT/GB 03/02832	

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	"STUDIES OF RESPIRATORY DISEASE IN RANDOM SOURCE LABORATORY DOGS VIRAL INFECTIONS IN UNCONDITIONED DOGS" BINN L N---; ALFORD J P; MARCHWICKI R H; KEEFE T J; BEATTIE R J; WALL H G, vol. 29, no. 1, 1979, pages 48-52, XP0008023128 cited in the application the whole document -----	
A	WO 93 23423 A (SMITHKLINE BEECHAM CORP ; JONES ELAINE V (US); KLEPFER SHARON (US); MI) 25 November 1993 (1993-11-25) the whole document -----	1-87
X	US 5 672 350 A (BABIU LORNE A ET AL) 30 September 1997 (1997-09-30) the whole document -----	47-54
A	WO 99 25838 A (CONTRERAS ANAPATRICIA ; BROWN THOMAS P (US); UNIV GEORGIA (US); VILLEG) 27 May 1999 (1999-05-27) claims 8-12 -----	1
A	US 6 057 436 A (KLEPFER SHARON ET AL) 2 May 2000 (2000-05-02) the whole document -----	1
A	EP 0 510 773 A (AKZO NV) 28 October 1992 (1992-10-28) the whole document -----	1
X	US 5 916 570 A (KAPIL SANJAY) 29 June 1999 (1999-06-29) the whole document -----	47-54
A	HASOKSUZ M ET AL: "ANTIGENIC VARIATION AMONG BOVINE ENTERIC CORONAVIRUSES (BECV) AND BOVINE RESPIRATORY CORONAVIRUSES (BRCV) DETECTED USING MONOCLONAL ANTIBODIES" ARCHIVES OF VIROLOGY, NEW YORK, NY, US, vol. 144, no. 12, 1999, pages 2441-2447, XP001005398 ISSN: 0304-8608 the whole document -----	18-46
P,X	ERLES KERSTIN ET AL: "Detection of a group 2 coronavirus in dogs with canine infectious respiratory disease." VIROLOGY, vol. 310, no. 2, 5 June 2003 (2003-06-05), pages 216-223, XP002257085 ISSN: 0042-6822 the whole document -----	1-7

INTERNATIONAL SEARCH REPORT

Intel' n al application No.
PCT/GB 03/02832

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION sheet PCT/ISA/210
2. Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 66 to 69 and 79 to 82 are directed to a method of treatment of the animal body, the search could be carried out and based on the alleged effects of the compound/composition.

Although claims 24 to 36, claims 67 to 69 and 87 are directed to a diagnostic method comprising steps practised on the animal body, the search has been carried out and based on the alleged effects of the compound/composition.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.5), should the problems which led to the Article 17(2) declaration be overcome.

INTERNATIONAL SEARCH REPORT

Information on patent family members

 Internal Application No
 PCT/GB 03/02832

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9323423	A	25-11-1993	AT 225805 T		15-10-2002
			AU 670092 B2		04-07-1996
			AU 4251593 A		13-12-1993
			CA 2135200 A1		25-11-1993
			DE 69332375 D1		14-11-2002
			DE 69332375 T2		13-02-2003
			DK 640098 T3		28-10-2002
			EP 0640098 A1		01-03-1995
			ES 2182827 T3		16-03-2003
			JP 3245169 B2		07-01-2002
			JP 7508421 T		21-09-1995
			PT 640098 T		31-01-2003
			WO 9323423 A1		25-11-1993
			US 6057436 A		02-05-2000
			US 6372224 B1		16-04-2002
			US 2002127245 A1		12-09-2002
US 5672350	A	30-09-1997	CA 2065365 A1		23-02-1991
			WO 9102752 A1		07-03-1991
			DE 69013512 D1		24-11-1994
			DE 69013512 T2		23-02-1995
			EP 0489033 A1		10-06-1992
			US 5369026 A		29-11-1994
WO 9925838	A	27-05-1999	AU 1408499 A		07-06-1999
			WO 9925838 A1		27-05-1999
US 6057436	A	02-05-2000	US 2002127245 A1		12-09-2002
			AT 225805 T		15-10-2002
			AU 670092 B2		04-07-1996
			AU 4251593 A		13-12-1993
			CA 2135200 A1		25-11-1993
			DE 69332375 D1		14-11-2002
			DE 69332375 T2		13-02-2003
			DK 640098 T3		28-10-2002
			EP 0640098 A1		01-03-1995
			ES 2182827 T3		16-03-2003
			JP 3245169 B2		07-01-2002
			JP 7508421 T		21-09-1995
			PT 640098 T		31-01-2003
			WO 9323423 A1		25-11-1993
			US 6372224 B1		16-04-2002
			AU 666298 B2		08-02-1996
			AU 9128691 A		11-06-1992
			CA 2096010 A1		17-05-1992
			EP 0557455 A1		01-09-1993
			IE 913948 A1		20-05-1992
			JP 6502768 T		31-03-1994
			JP 2003093078 A		02-04-2003
			KR 204258 B1		15-06-1999
			NZ 240558 A		25-11-1994
			WO 9208487 A1		29-05-1992
			US 6280974 B1		28-08-2001
			US 2002115064 A1		22-08-2002
			ZA 9108988 A		30-12-1992
EP 0510773	A	28-10-1992	AU 645417 B2		13-01-1994
			AU 1513192 A		29-10-1992

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internal Ref.	Application No
PCT/GB 03/02832	

Patent document cited in search report	Publication date	Patent family member(s)			Publication date
EP 0510773	A	CA	2066940 A1		26-10-1992
		EP	0510773 A1		28-10-1992
		JP	3420258 B2		23-06-2003
		JP	5260976 A		12-10-1993
		NZ	242471 A		26-05-1993
		US	5661006 A		26-08-1997
-----	-----	-----	-----	-----	-----
US 5916570	A 29-06-1999	AU	705186 B2		20-05-1999
		AU	6383296 A		09-01-1997
		CA	2224309 A1		27-12-1996
		EP	0833903 A1		08-04-1998
		WO	9641874 A1		27-12-1996
-----	-----	-----	-----	-----	-----